Novel techniques for the incorporation of proteins in biodegradable polymeric drug delivery devices for their controlled release

Dissertation
zur
Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)

dem
Fachbereich
der Philipps-Universität Marburg
vorgelegt von

Sascha Maretschek

aus Fulda

Marburg an der Lahn 2009

Vom Fachbereich Pharmazie der Philipps-Universität Marburg als Dissertation am 23.03.2009 angenommen.

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Tag der mündlichen Prüfung am 29.04.2009

Die vorliegende Arbeit entstand
auf Anregung und unter Leitung von

Herrn Prof. Dr. Thomas Kissel

am Institut für Pharmazeutische Technologie und Biopharmazie
der Philipps-Universität Marburg.

Meiner Familie

In Liebe und Dankbarkeit

Danksagung

Mein besonderer Dank gilt Herrn Prof. Dr. Thomas Kissel für die Betreuung meiner Doktorarbeit, die Freiheit bei der Umsetzung sowie die stete Unterstützung und Ermutigung während der Durchführung. Sein Vertrauen in den Erfolg meiner Themen und seine stete Diskussionsbereitschaft während der gesamten Promotion, sowie die ausgesprochen angenehme Atmosphäre in unserem Arbeitskreis haben maßgeblich zum Gelingen dieser Arbeit beigetragen. Er war stets ein verständnisvoller und motivierender Doktorvater für mich und hat es mir ermöglicht, sehr innovative Themen bearbeiten zu dürfen und mit Arbeitsgruppen anderer Fachbereiche zusammen arbeiten zu können.

Prof. Dr. Udo Bakowsky möchte ich für die Erstellung des Zweitgutachtens sowie seine guten Ratschläge, seine Diskussionsbereitschaft und viele ermutigende Gespräche danken.

Prof. Dr. Andreas Greiner danke ich dafür mir die Möglichkeit gegeben zu haben, die Elektrospinning-Apparaturen in seinem Arbeitskreis nutzen zu dürfen.

Für die stete Unterstützung während meiner ersten Zeit im Arbeitskreis danke ich meinen ehemaligen Kollegen Dr. Matthias Wittmar und Dr. Holger Petersen.

Für die Unterstützung bei der Einarbeitung im Bereich Electrospinning möchte ich den ehemaligen Mitgliedern des AK Greiner Dr. Holger Wickel, Dr. Matthias Becker und Dr. Olaf Kriha danken.

Nina Seidel möchte ich für ihre große Hilfe bei der Erstellung von REM Bildern, ihre stete Diskussionsbereitschaft sowie ihre Unterstützung in allen Lebenslagen von ganzem Herzen danken.

Ganz besonderer Dank gebührt Dr. Claudia Packhäuser und Dr. Martina Lehmann, die mich schon vom Beginn des Studiums an über die Diplomarbeit bis hin zur Dissertation in Marburg begleitet und unterstützt haben.

Oliver Germershaus danke ich besonders für seine Unterstützung bei der Durchführung der Zellversuche sowie für die Erstellung der CLSM Bilder.

Tobias Lebhardt möchte ich dafür danken, dass er mir besonders am Ende meiner Promotionszeit viel Arbeit im Bereich EDV- und Netzwerkbetreuung abgenommen hat.

Die vielen schönen Stunden mit meinen Kollegen Dr. Michael Neu, Dr. Juliane Nguyen, Dr. Terry Steele, Dr. Erik Rytting, Dr. Yu Liu, Frank Morell, Olivia Merkel, Regina Reul, Nan Zhao, Christoph Schweiger, Heiko Debus, Markus Benfer, Nadja Bege, Thomas Renette, Moritz Beck-Broichsitter und Susanne Rösler während und nach der Arbeit, werden mir für immer angenehm in Erinnerung bleiben. Gleiches gilt für die Kollegen aus dem Arbeitskreis von Prof. Bakowsky: Anett Sommerwerk, Nico Harbach, Mario Bandulik, Jens Schäfer, Eyas Dayyoub, Aybike Özcetin, Elena Marxer und Thomas Betz sowie Johannes Sitterberg, der außerdem auch rasterkraftmikroskopische Untersuchungen für diese Arbeit durchführte.

Weiterhin gilt mein Dank Eva Mohr, für ihre stete Hilfsbereitschaft im Bereich der Zellkultur und Klaus Keim für die Anfertigung von hervorragenden REM-Bildern.

Ein großes Dankeschön geht auch an meine Familie für all ihre Unterstützung. Ganz besonders möchte ich meinen liebevollen Eltern für ihre Hilfe in allen Lebenslagen und ihr Verständnis für all meine Entscheidungen von ganzem Herzen danken.

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INTRODUCTION

Objectives of this work:

In this dissertation two novel technologies for the incorporation of proteins in a biodegradable drug delivery device for their controlled release are presented. In the first part of this thesis, electrospinning of emulsions composed of an organic polymer solution and an aqueous protein solution is illustrated as a versatile technique to generate protein loaded nanofiber nonwovens. After that, a new microencapsulation technique based on ionic liquids and biodegradable polymers is presented. With the recent progress in biotechnology and genetic engineering a large number of protein drugs have found therapeutic application. Futhermore, a prolonged delivery of bioactive proteins is also desirable for several biomedical applications like tissue engineering. In most cases these bioactive agents are rapidly degraded and deactivated. Due to their short biological half-lives, frequent administration is needed to achieve adequate concentrations for prolonged periods of time. Biodegradable delivery systems, such as nanofiber nonwovens or microparticles have proven their suitability as protein delivery devices for their use in biomedical applications.

Chapter 1 gives an overview on the basic principles of the standard electrospinning process, the equipment needed and important process parameters. Several modifications of this technique are also discussed as well as the suitability of the resulting nanofibrous scaffolds for biomedical applications.

For a successful preparation of protein loaded nanofiber nonwovens proper materials have to be selected. Several biodegradable polymers and the according suitable process parameters for the electrospinning of an emulsion of an aqueous cytochrome C solution in the respective polymer solution were investigated in **Chapter 2**. The resulting nanofiber nonwovens were characterized regarding their morphology and their protein release profile to determine the best candidate for subsequent studies.

In **Chapter 3** electrospinning of emulsions composed of an organic poly(L-lactide) solution and an aqueous cytochrom C solution is described in detail. Different polymer concentrations and blending with hydrophilic polymers were illustrated and the effects on morphology and the protein release characteristics were demonstrated.

A scaffold has to meet certain key requirements for its application in tissue engineering. **Chapter 4** deals with the interactions of cells with nanofiber nonwovens composed of different polymers and polymer blends and clarifies whether the manufactured nanofibrous scaffolds met the requirements for a tissue engineering scaffold.

An overview on microencapsulation focusing on phase separation and coacervation techniques is given in **Chapter 5.** The development of these techniques and its application in the pharmaceutical industry are elucidated.

Ionic liquids are a new class of solvents with remarkable characteristics. **Chapter 6** deals with the development of a new microencapsulation technique which uses an ionic liquid as one common solvent for a biodegradable polymer and a hydrophilic macromolecule. The solubilities of several biodegradable polymers and hydrophilic macromolecules in ionic liquids were determined and a suitable technique for the microencapsulation of bovine serum albumin and several dyes was developed.

Chapter 1:

A short overview on electrospinning of nanofiber nonwovens for biomedical applications

Electrospinning is a term used to describe a class of fiber forming processes by which electrostatic forces are employed to control the production of fibers. It is a unique technology for the production of nanofiber nonwovens with fiber diameters ranging from tens of nanometers to microns, a size range that is otherwise difficult to access by conventional non-woven fiber fabrication techniques [1-3]. Electrospinning first appeared in the patent literature in 1902 [4], but the technology did not receive notable attention (apart from the studies by Baumgarten [5] and Larrondo [6-8]) until the 1990s when Reneker and co-workers described electrospinning as a technique for the production of small diameter, continuous filaments [3, 9, 10].

Electrospun nanofiber scaffolds possess high surface-to-volume ratios, adjustable porosities and can be produced in a wide variety of sizes and shapes. In addition, the scaffold composition can be controlled to achieve desired properties and functionality. Due to these advantages, electrospun nanofibrous scaffolds were widely investigated in the past years with materials of different compositions [11-15], for various applications, such as filtration [16-18], optical and chemical sensors [19-24] and tissue engineering [25-28].

On a laboratory scale, electrospinning is a simple method to generate nanoscale fibers. Figure 1 shows schematically an electrospinning system, which usually consists of three components: a high voltage supply, a spinneret (usually a metallic capillary) and a grounded collecting plate (usually a metal plate or a rotating drum) [29]. The spinneret acts simultaneously as an electrode, to which high voltage of 10 – 30 kV is applied. The collecting plate acts as a counter electrode with a distance between the two electrodes of ca. 10 – 25 cm. The amperage during electrospinning ranges from a few hundred nanoamperes to microamperes.

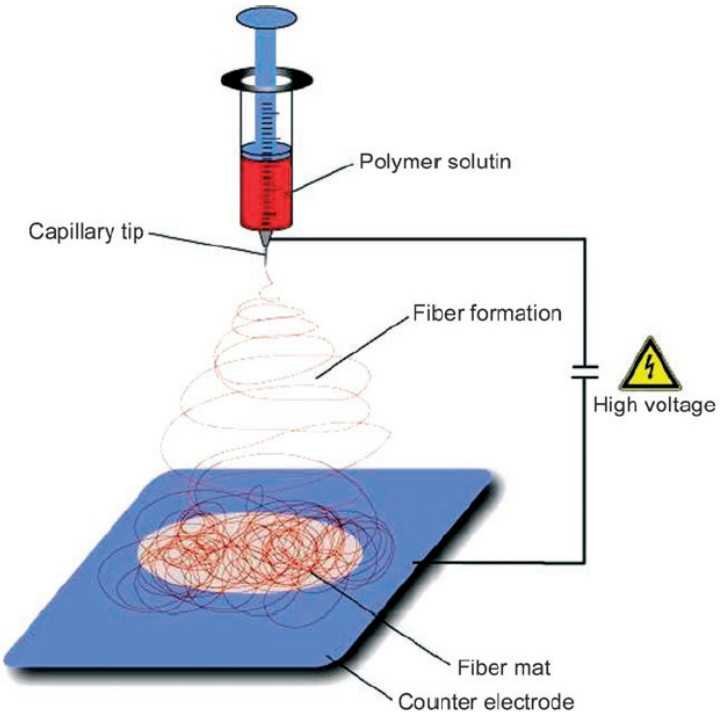

Figure 1: Basic electrospinning instrumentation consisting of a high voltage supply, a spinneret and a grounded collecting plate.

When a polymer solution or melt is fed through the spinneret the electric field leads to a conical deformation of the droplet shape, which is designated "Taylor cone" after the pioneering work of Sir Geoffrey Ingram Taylor in 1964 [30]. He theoretically derived, that the perfect cone under such conditions has an angle of 98.6°. Beyond a critical charge density, the electrostatic repulsion of the charges overcomes the surface tension of the droplet, the cone becomes unstable and a jet of fluid is emitted from the apex of the cone. Figure 2 shows the conical deformation of a droplet in an electrical field [31].

Figure 2: Deformation of a droplet in an electric field with increasing field strength leading to the formation of the so called "Taylor cone".

During the jet propagation towards the counter electrode the solvent in the jet stream evaporates and the fiber solidifies. As it does so, the filament is accelerated, stretched and may experiences a number of instabilities. In principle, the cone-jet operation is sufficient to draw out continuous fibers of small diameters by electrospinning. However in practice, as the jet thins, it usually undergoes one or more fluid instabilities which distort the jet as they grow. Although splaying of the jet was proposed in the 1990's as the primary mode of instability [9], such events are relatively rare. The most common mode of instability in electrospun jets appear to be the growth of lateral excursions of the jet, the so called whipping instabilities [32-36].

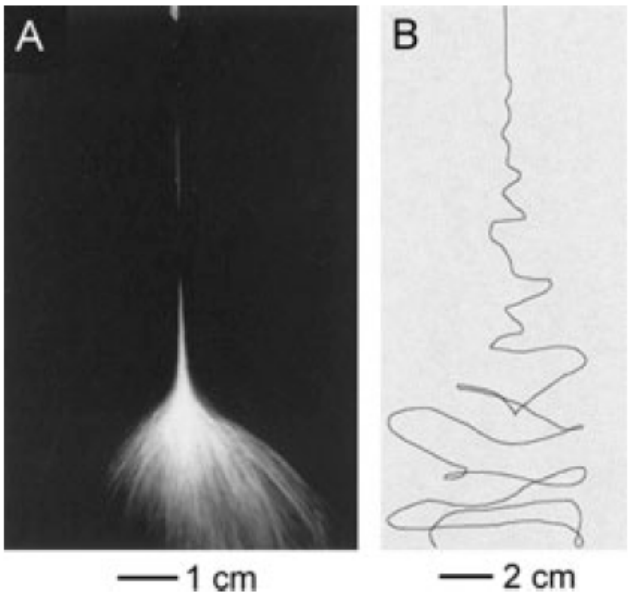

Figure 3: Jet instabilities during electrospinning (A: real time image, B: high speed image)

Figure 3 illustrates the instability region of a liquid jet electrospun from an aqueous solution of poly(ethylene oxide). The first picture resembles the view of the jet in real time, demonstrating that the jet at first followed a direct path to the counter electrode and subsequently became instable. It appears that the cone-shaped instability region is composed of multiple jets. However, the second picture shows a high speed photograph of the jet, which proves that the conical envelope contained only a single, rapidly bending or whipping thread.

These bending instabilities are mainly caused by electrostatic interactions between surface charges on the jet and the electric field and are the main reason for the high stretching rates which can be achieved by electrospinning [34, 36].

The resulting product is a nonwoven fibrous scaffold with a large surface area-to-volume ratio and small pore sizes. Fiber diameter and morphology can be controlled by many parameters, such as solution properties (viscosity, elasticity, conductivity and surface tension), electric field strength, distance between the spinneret and the collecting plate, temperature and humidity [37]. Due to the small fiber diameters, the yield per spinneret of the electrospinning process is extremely low. Recently, multi-jet electrospinning (Figure 4a) [38-40], blowing assisted electrospinning [41] and needleless upward electrospinning technologies (Figure 4b) [42] were developed, demonstrating the production capability for fabricating nanofibrous scaffolds in an industrially relevant scale.

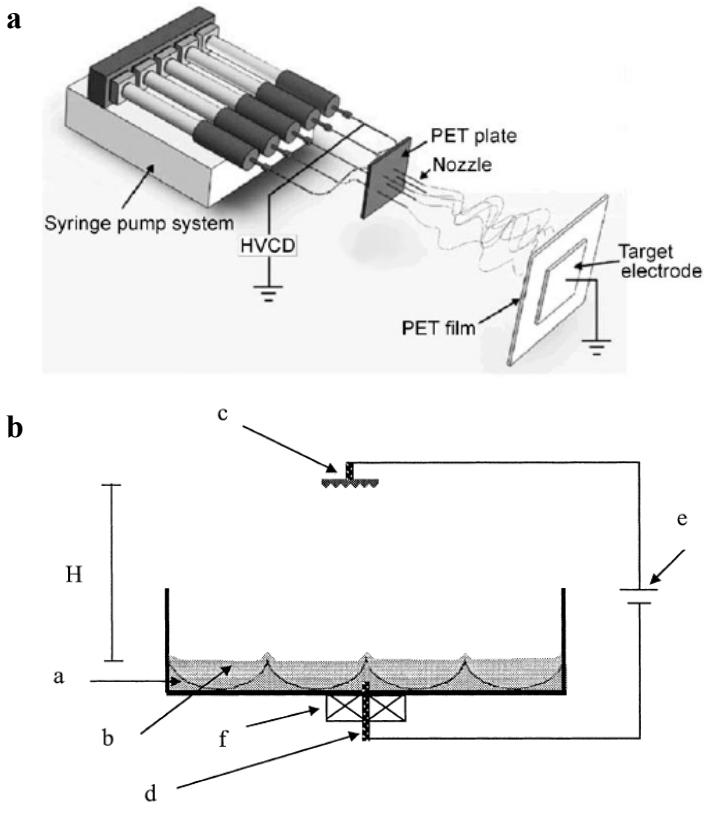

Figure 4: New technologies for the preparation of nanofiber nonwovens in an industrially relevant scale. (a: multi-jet electrospinning, b: needleless upward electrospinning)

The use of electrospun nanofibrous scaffolds for biomedical applications has attracted a great deal of attention in the past several years. Nanofibrous scaffolds have demonstrated their suitability as tissue engineering scaffolds [43-49], immobilized enzymes and catalysts [50-57], as wound dressings [14, 58-63] and artificial blood vessels [64-68]. They have also been used as barriers for the prevention of post-operative induced adhesion [69-71] and vehicles for drug delivery [72-78]. For a successful application the nanofibrous scaffold has to exhibit suitable physical and biological properties closely matching the desired requirements. For example, in tissue engineering, the electrospun scaffold should physically resemble the nanofibrous features of the extracellular matrix (ECM) with suitable mechanical properties. It should also promote cell adhesion, spreading and proliferation. For a wound dressing, the nanofibrous scaffold should not only serve as a substrate for tissue regeneration, but could also deliver bioactive agents or antibiotic drugs in a controlled manner during the healing process.

Physical and biological properties of electrospun nanofibrous scaffolds for biomedical applications such as hydrophilicity, mechanical modulus and strength, biodegradability, biocompatibility and specific cell interactions are largely determined by the materials' chemical composition. Based on polymer physics, copolymerization and polymer blending are two effective means to combine different polymers to yield new materials properties. Thus, by selecting a combination of proper components and by adjusting the components ratio, properties of electrospun scaffolds can be tailored to the desired functions. For example, many kinds of copolymers [79-84] and polymer mixtures [85-95] were electrospun for biomedical applications with varying degrees of success.

Many biopolymers like proteins, polysaccharides, DNA and lipids were also electrospun into fibrous scaffolds. Protein fibers, especially composed of collagen, gelatin, elastin and silk fibroin, were intensively studied in recent years [14, 64, 96-105]. Electrospun collagen

nanofiber scaffolds are particularly interesting for tissue engineering applications, since collagen is the principal structural element of the extracellular matrix in tissues. In the biopolymer class of polysaccharides dextran, chitosan and cellulose acetate were successfully electrospun into nanofibrous scaffolds. Electrospinning of aqueous hyaluronic acid solutions was also possible via a "blowing-assisted" electrospinning process [41]. These solutions had to be electrospun with the assistance of air flow at elevated temperatures. Nanofiber nonwovens composed of hyaluronic acid with a suitable degree of post-crosslinking are suitable scaffolds for cartilage repair, since hyaluronan is an abundant polysaccharide found almost exclusively in articular joints. Apart from proteins and polysaccharides, calf thymus DNA in an aqueous solution was electrospun to form nanofibers with diameters ranging from 30 – 80 nm [106, 107]. However, no specific biomedical applications of such DNA nanofibers have been reported. Recently, McKee et al. reported nonwoven membranes electrospun from lecithin solutions in a single processing step [108].

Besides taking advantage of the materials compositions, the fabrication process, through which the fiber diameter, morphology and scaffold porosity can be manipulated, also plays an important role on the scaffold properties and functionality. Several innovative electrospinning techniques can enhance the functions and properties of electrospun nanofibers.

Since the velocity of the fiber jet near the counter electrode is very high, the nanofibers are usually collected in a random fashion yielding nanofiber nonwovens. However, several fiber collection methods were described to obtain aligned nanofibers. Probably the most common technique relies on a rapidly rotating cylindrical collector, which either serves as counter electrode or is combined with an electrode [109-112]. Parallel fibers can also be produced with special electrode arrangements [113, 114]. The application of orientated electrospun scaffolds in the biomedical field was demonstrated in several studies. Xu et al. [68] investigated an aligned nanofibrous scaffold and found that human coronary artery smooth

muscle cells attached and migrated along the axis of the aligned nanofibers. They also found that adhesion and proliferation rate of the cells on the nanofibrous scaffold were significantly improved compared to those on a solid polymer film.

The fabrication of core-shell nanofibers by coaxial electrospinning was first reported by Sun et al. [115]. This technology provided the possibility to co-electrospin polymer solutions, which where difficult to process in a standard electrospinning process, yielding an ultrafine core within the shell of other polymeric materials (Figure 5). Furthermore, the coaxial electrospinning process provided a new pathway to incorporate drugs inside a fiber core, yielding nanofibers which were able to release the drugs in a controlled manner over a prolonged period of time [73, 116-120].

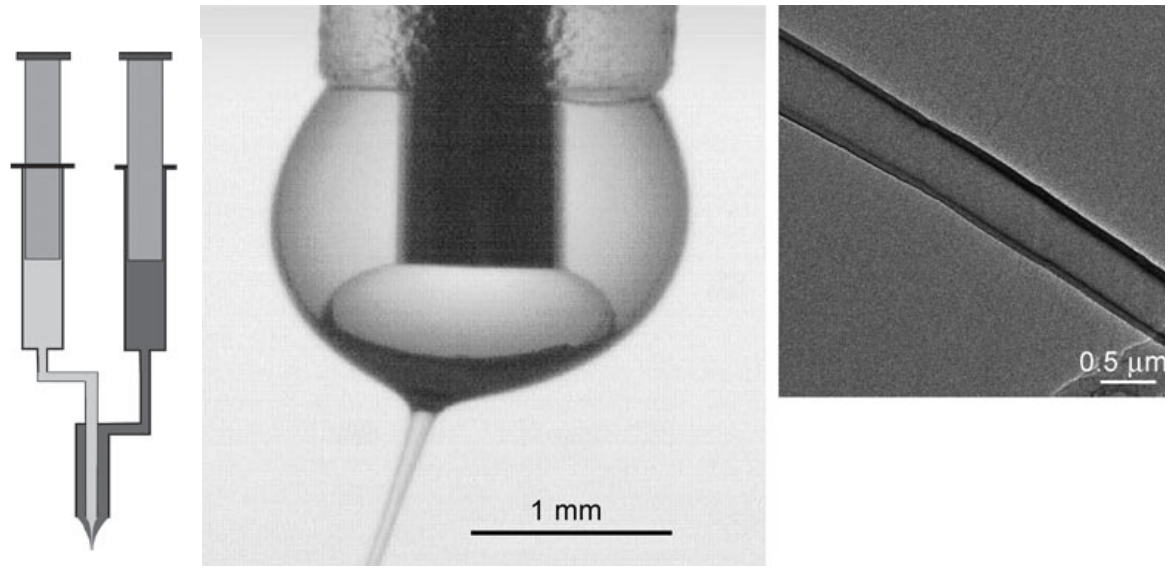

Figure 5: Principle of coaxial electrospinning and the resulting core-shell fibers.

In contrast to coaxial electrospinning, where two different solutions are electrospun without direct mixing, two phase electrospinning describes electrospinning of two immiscible solutions as an emulsion [121-125]. The two-phase electrospinning process, using a single spinneret, provides the possibility to incorporate small molecules and/or macromolecules, including drugs and proteins in the nanofibrous scaffolds. The encapsulated bioactive molecules can be immobilized for a long time and afterwards released in a controlled manner.

Conclusion:

Electrospun nanofibrous scaffolds demonstrated great potential for many biomedical applications. The concept of two-phase electrospinning offers the possibility of incorporating hydrophilic macromolecules in biodegradable nanofiber nonwovens for their controlled release. Due to its simplicity, this technique may offer potentially useful advantages over other electrospinning techniques in the applications of drug delivery and tissue engineering. A successful creation of nanofiber nonwovens has to start with the proper selection of materials. Chapter 2 will describe suitability studies of several biodegradable polymers for the fabrication of protein loaded nanofiber nonwovens. After a suitable candidate has been selected, mixtures of this biodegradable polymer with other polymers might be a useful approach to adjust the properties of the nanofiber nonwoven. This topic will be elucidated in Chapter 3. Whether the prepared drug delivery devices are suitable tissue engineering scaffolds will be demonstrated in Chapter 4 by investigating the interactions of a model cell line with the prepared nanofiber nonwovens.

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Chapter 2:

Evaluation of suitable polymers and process parameters for the generation of protein loaded nanofiber nonwovens by electrospinning

Summary:

Several biodegradable polymers (Poly(L-lactide) (PLLA) Resomer L210, poly(D,L-lactide-co-glycolide) (PLGA) Resomer RG858 and Poly(ethylene carbonate) (PEC)) were investigated for their suitability as biomaterials for the electrospinning of emulsions. Suitable process parameters were determined empirically and the resulting nanofiber nonwovens (NNs) were examined regarding their morphology before and after incubation in buffer solutions and regarding their protein release profile. PEC formed the most uniform fibers with diameters of 1 – 5 µm. PLLA and PLGA formed less uniform fibers but with smaller diameters of 0.3 to 1 µm. PEC and PLGA NNs showed only burst release of the incorporated protein and no further release within 2 weeks. PLLA fiber released the protein very slowly and in a controlled manner. Due to swelling PLGA and PEC NNs showed tremendous changes in morphology after the release study. The morphology of PLLA NNs was more or less unchanged after the release study.

Introduction:

Polymeric drug delivery devices have numerous advantages compared to conventional dosage forms, such as improved therapeutic effect, reduced toxicity and convenience. However, the conventional polymeric drug delivery systems like nano- or microspheres, liposomes and hydrogels often exhibit the problem of a high burst release at the beginning. Electrospinning is a versatile technique for the generation of drug loaded polymeric nanofiber nonwovens for the controlled release of the incorporated drugs [1-6]. During the electrospinning process a strong electrostatic field is applied to a polymer solution held in a syringe with a capillary metal outlet. A pendant droplet of the polymer solution from the capillary outlet is deformed into a Taylor cone by the electrostatic field [7]. When the voltage surpasses a threshold value, the electric force overcomes the surface tension of the droplet and a charged jet of the solution is ejected from the apex of the Taylor cone [8, 9]. The fibers are collected as a nonwoven mat on an electrically grounded target.

Encapsulation of low molecular weight drug substances like tetracycline hydrochloride [10], ibuprofen [11], rifampicin [12], and other molecules into electrospun NNs was already demonstrated by a number of authors [6, 13-17]. In most cases the drug was dissolved or dispersed in the polymer solution and was encapsulated using a one phase electrospinning method. Only very few studies on the encapsulation and release of macromolecules like proteins have been published [18-21]. Encapsulation of higher molecular weight substances is more difficult, because the electrospinning of macromolecular dispersions is more challenging than electrospinning of homogenous polymer solutions. The concept of coaxial electrospinning led to core-shell structured fibers which proved their suitability as drug delivery devices for larger molecules, but special equipment is needed for these electrospinning techniques [22-24]. Another approach which has not been investigated very extensively until now is the electrospinning of emulsions [19, 21, 25, 26].

The goal of this study was the evaluation of suitable polymers and process parameters for the generation of protein loaded nanofiber nonwovens by electrospinning of emulsions. Poly(L-lactide) and poly(D,L-lactide-co-glycolide) were chosen for this study, since they are the most widely studied biomaterials for protein drug delivery. Futhermore, poly(ethylene carbonate) was investigated for its suitability, since this polymer has very promising properties for its application in drug delivery. Cytochrome C was used as a model compound for the encapsulation of proteins. Suitable process parameters for the electrospinning of W/O emulsions of those polymer solutions were determined empirically and the morphology and the release profiles of the resulting nonwovens were investigated.

Materials and methods:

Poly(L-lactide) (PLLA) Resomer L210 (inherent viscosity 3.3 – 4.3 dl/g) and poly(D,L-lactide-co-glycolide) (PLGA) Resomer RG858 (inherent viscosity 1.3 – 1.7 dl/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(ethylene carbonate) (PEC, batch 96906) was synthesized and provided by Novartis Pharma AG (Basel, Switzerland). Cytochrome C from horse heart was obtained from Fluka Chemie GmbH (Buchs, Germany). All other reagents used were of analytical grade.

Electrospinning

Different emulsions were prepared in 5 ml syringes using an Ultra Turrax T25 running at 20500 rpm for 1 minute. A setup published previously was used for electrospinning [27]. Electrospinning was carried out using a variable high-voltage power supply, which can produce voltages ranging from 0 to 55 kV. It consists of one positive power supply attached to a blunt steel needle and one negative power supply attached to the counter electrode. The syringes containing the different emulsion were attached to the blunt needle and were put into

a syringe pump pushing 50 µl of the emulsions per minute through the blunt needle. A round steel plate with a diameter of 9 cm served as the counter electrode. It was covered with aluminium foil for the collection of the different fibers.

Nanofiber nonwovens were prepared by emulsifying 0.5 ml of an aqueous solution of Cytochrome C (20 mg/ml) into 2 g of different polymer solutions in methylene chloride. A voltage of +19 kV was applied at the needle tip, and –1 kV was applied at the counter electrode, which was 20 cm from the needle tip.

SEM imaging

Fragments of the nanofiber nonwovens were sputtered with gold in an argon atmosphere (0.3 mPa) using a sputter coater S-150 (Edwards Kniese & Co. GmbH, Marburg, Germany). Sputtering was carried out for each sample at 18 mA 2 times for 1 minute and after that 2 times for 2 minutes. Images were taken with an SEM S-510 at 25 kV accelerating voltage (Hitachi Ltd. Corporation, Tokyo, Japan) using the Digital Imaging Scanning System (Version 5.4.12.1) purchased from point electronic GmbH, Halle (Saale), Germany. Images were analyzed with the Digital Image Processing System (Version 2.6.13.0) purchased from point electronic GmbH, Halle (Saale), Germany.

AFM imaging

AFM pictures were recorded using a JPK NanoWizard™ (JPK Instruments, Berlin, Germany) in intermittent contact mode to prevent sample damage. Si_3N_4 tips attached to I-shaped cantilevers with a length of 125 µm were used. The cantilevers had a resonance frequency of 160 kHz. The line scan speed was about 1 Hz. Images were obtained in trace direction for amplitude displaying pictures and in retrace direction for height pictures, with both signals

being recorded simultaneously. Images were recorded in the height, amplitude and phase mode. For data analysis, the producer's software was used.

Protein Stability

Native Page gel electrophoresis experiments were carried out using a Pharmacia LKB PHAST system (Pharmacia Biosciences, Uppsala, Sweden) and PhastGel separation media for Native PAGE (Pharmacia Biosciences, Uppsala, Sweden). The different protein solutions were applied on the ready-to-use PhastGel 18-25 (Pharmacia Biosciences, Uppsala, Sweden) via a PhastSystem applicator comb. These special sample applicators ensured that identical volumes were put on the separation gel. PhastGel Native ready-to-use buffer strips were used for the separation process. After applying current, the proteins migrated through the gradient gel and were separated according to their size. A Coomassie staining was carried out for protein detection using PhastGel Blue R tablets.

Loading

Loading of PEC nanofiber nonwovens was determined by dissolution of the fibers in methylene chloride and extraction of the protein with water. Loading of PLLA and PLGA nanofiber nonwovens was determined by degrading parts of the nanofiber nonwovens in 1 ml of 1 N NaOH. The amount of incorporated Cytochrome C was calculating by measuring the adsorption of the resulting solution at 412 nm using a UV/Vis spectrophotometer UV-160 (Shimadzu Corporation, Kyoto).

Release profile

Release studies were carried out in PBS buffer solution pH 7.4. Samples of the nanofiber nonwovens (2 cm x 2 cm) were put in 15 ml plastic tubes and 2 ml release medium was

added. The tubes were constantly moved at a speed of 30 rpm at 37°C. At certain time points 200 µl of the release medium were removed and replaced with fresh buffer solution. The amount of released cytochrome C was determined via BCA assay. 50 µl of the samples were mixed with 170 µl BCA reagent (BCA assay kit, Pierce, Rockford IL, USA) in 96 well plates. After 18h at ambient temperature the absorption at 560 nm was measured using a plate reader. All measurements were carried out at least in triplicate.

Statistical analysis

All quantitative results were carried out at least in triplicate. Data is expressed as mean ± SD. Statistical analysis was carried out using an unpaired student's t-test. A value of $p < 0.05$ was considered to be statistically significant.

Results and discussions:

The objective of this study was to evaluate the feasibility to generate biodegradable nanofiber nonwovens (NN) via electrospinning of emulsions for the controlled release of proteins. The most widely investigated class of biodegradable polymers with regard to toxicological and clinical data are aliphatic poly(ester)s consisting of lactic and glycolic acid. Their corresponding polymers, poly(lactide) (PLA), poly(glycolide) (PGA) and copolymer poly(lactic-co-glycolic acid) (PLGA) have found wide-spread commercial application as drug delivery devices. Poly(ethylene carbonate) (PEC) is a thermoelastic polymer of high-molecular weight which biodegrades non-hydrolytically and exclusively by surface erosion [28-31]. These degradation characteristics make PEC especially useful as a polymer matrix delivery system for the controlled release of proteins. We chose three different polymers for the evaluation of their suitability to prepare protein loaded NNs via electrospinning of emulsions: PEC (provided by Novartis), PLGA (RG858) and PLLA (Resomer L210).

Electrospinning is a very complex process, which is influenced by many different factors like the properties of the polymer itself (e.g. molecular weight, molecular weight distribution, glass transition temperature, and soluibility) and its solution (e.g. viscosity, concentration, surface tension, electrical conductivity). Even relative humidity and ambient temperature can have a significant impact. For these reasons, it is hard to make general recommendations about the process parameters and they usually have to be determined empirically. The following process parameters or ranges thereof where found to be suitable for electrospinning of the emulsions of this study: The distance between needle tip and counter electrode was always 20 cm and the voltage ranged between 20 to 25 kV. The feed rate of the emulsions was kept constant at 3 ml/h. The viscosities of the different emulsions were quite similar and the viscosity of an emulsion with a 2% PLLA solution as organic phase was determined as an example using a rotary viscosimeter. The experiment demonstrated that the emulsion was a Newtonian fluid with a viscosity of 921 mPa · s.

After suitable process parameters were determined, emulsions of different compositions were electrospun and the morphologies of the resulting NNs were analyzed via SEM imaging. The PEC fibers adhered very strongly to the aluminium foil, which was used for their collection. As a consequence, the NNs were highly stretched during removal from the aluminium foil. PEC is known for its very high stretching rates [32]. Figure 1 shows SEM images of PEC NNs on aluminium foil (a and b) and PEC NNs after removal from the aluminium foil (c and d). There were no visible changes in morphology after the removal. NNs consisting of PEC showed the most uniform fibers but also the largest fiber diameters ranging from 1 to 5 µm.

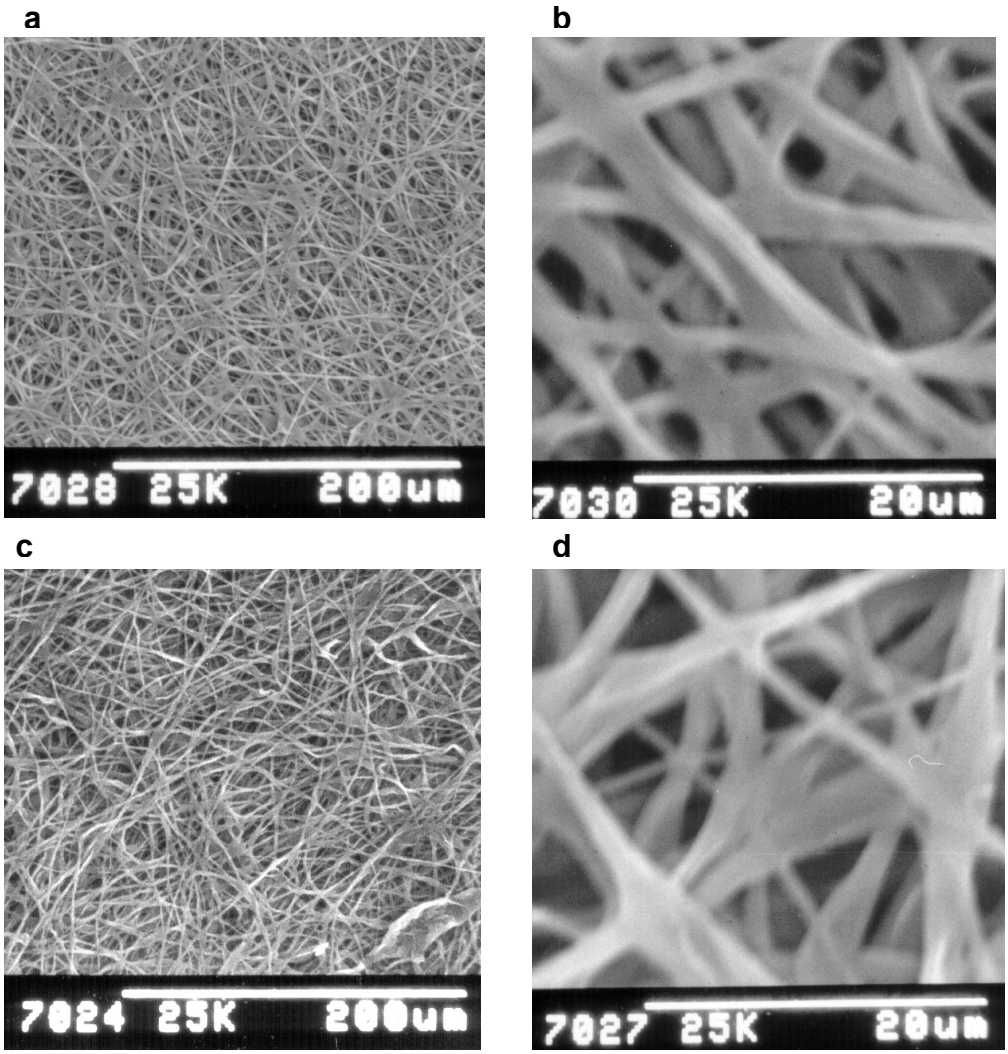

Figure 6: SEM images of PEC nanofiber nonwovens on aluminium foil (a and b) and after removal from the aluminium foil (c and d).

We were also able to visualize PEC NNs using AFM microscopy. AFM images confirmed that the fiber diameters ranged from 1 to 5 µm and furthermore demonstrated the smooth and nonporous surface of the PEC fibers (Figure 2).

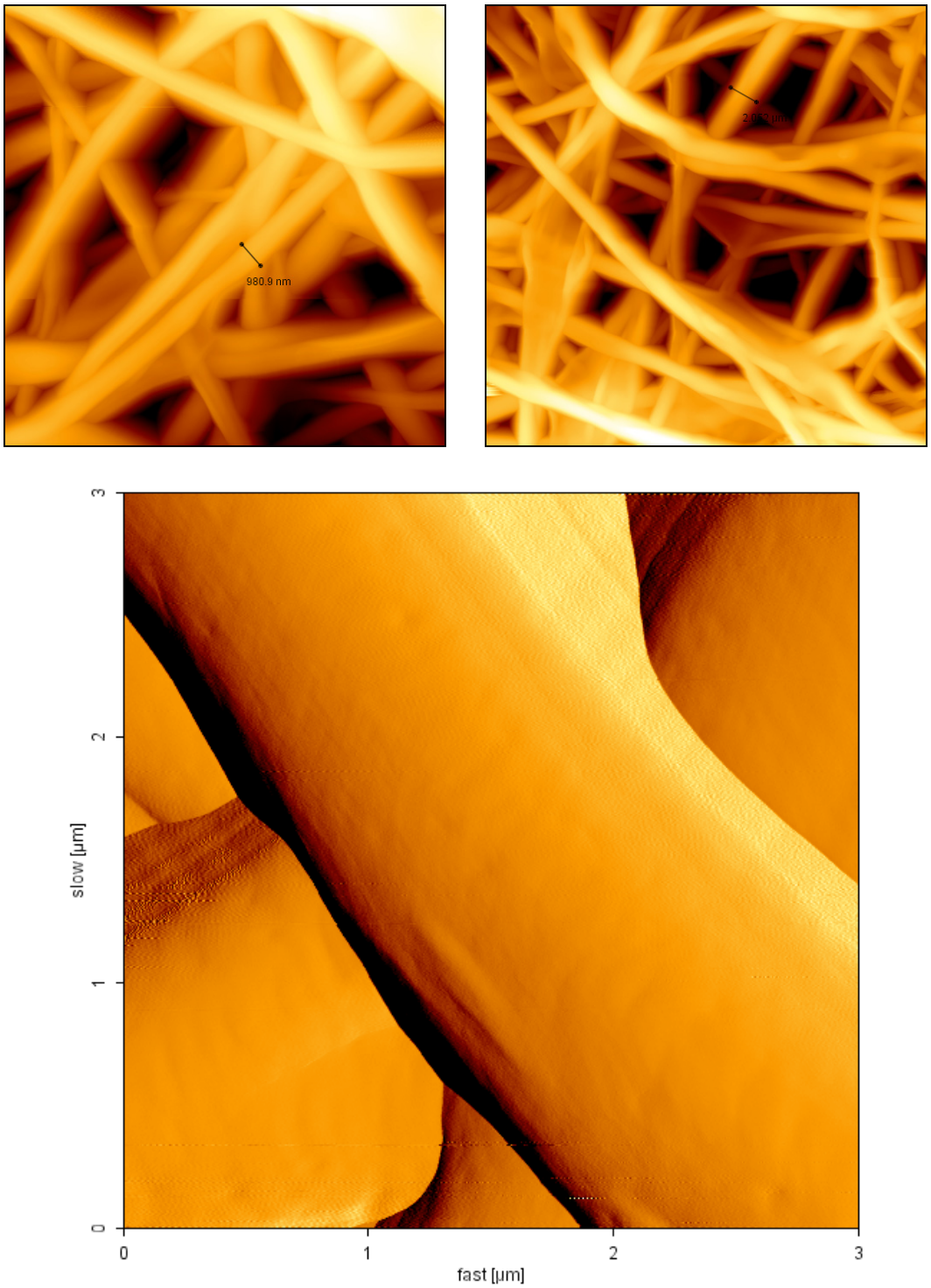

Figure 7: AFM images of PEC nanofiber nonwovens.

PLGA NNs consisted of rather irregular fibers and some bigger structures, which looked like burst beads. These structures are most likely due to the formation of a polymeric skin on the liquid jet covering the droplets of the aqueous phase at first and then collapsing (Figure 3).

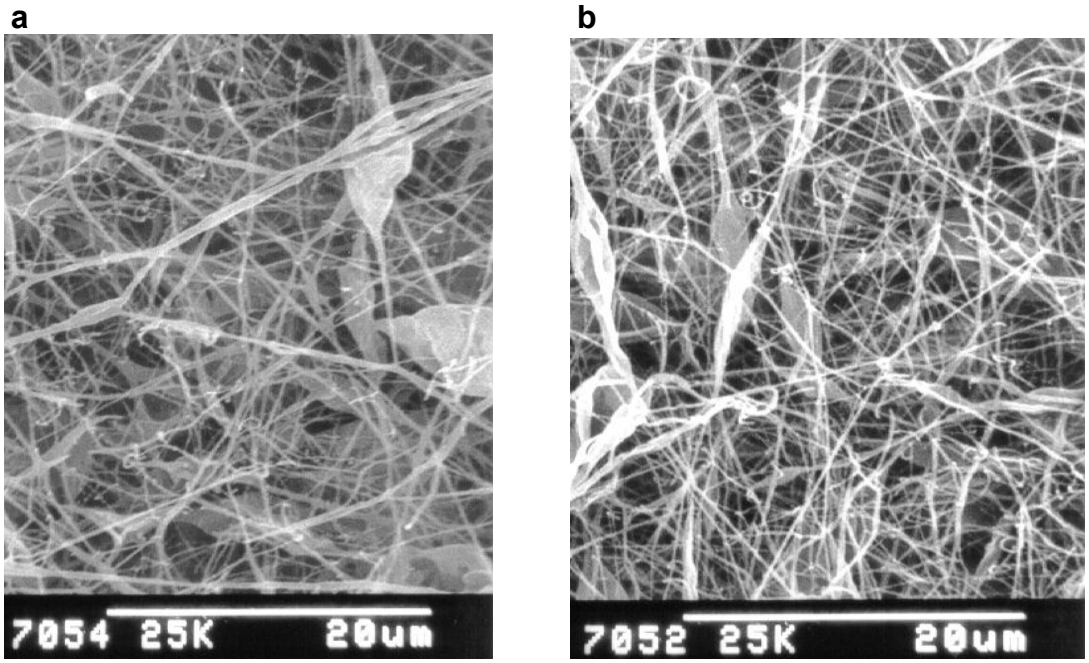

Figure 8: SEM images of PLGA nanofiber nonwovens.

Depending on the concentration of polymeric solution these structures could also be found in PLLA NNs. Higher polymer concentrations in the organic phase led to more uniform fibers with bigger diameters and less burst bead structures (Figure 4). In summary, PLLA NNs showed the most promising morphology, since non-fibrous structures only occurred at lower polymer concentrations and even at higher concentrations fiber diameters where still in a nanometer scale.

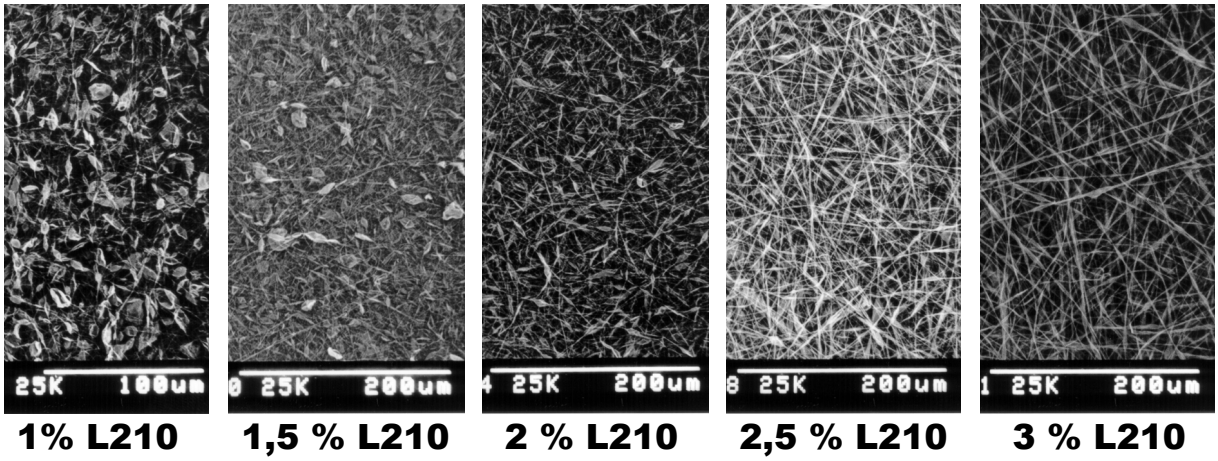

Figure 9: SEM images of PLLA nanofiber nonwovens.

Native page gel electrophoresis was used to check whether the electrospinning process would have a negative effect on the protein. Rather high concentrations of the protein had to be applied on the gel, to achieve a visible Coomassie staining. Nevertheless, there were no visible changes in retention time between native cytochrome C, a cytochrome C solution, which was kept at 37 °C for 2 days, and the cytochrome C released from PLLA NNs. These results proved that neither degradation nor aggregation of the protein occurred during electrospinning (Figure 5). Other groups have also shown that electrospun proteins are stable and retain their bioactivity [18, 20].

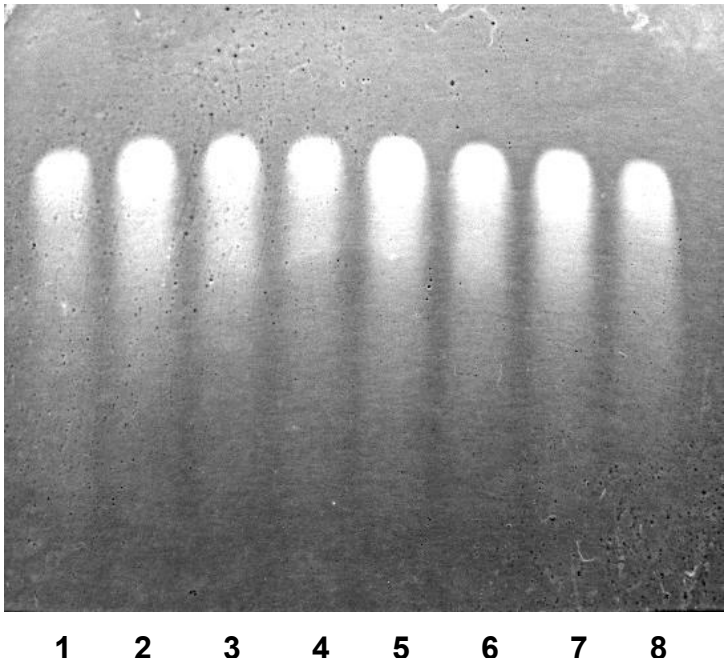

Figure 10: Native page gel of cytochrome C solutions (lane 1,4: fresh CytC solution as reference, lane 2, 5, 7: CytC released from PLLA nanofiber nonwovens, lane 3, 6, 8: CytC solution kept at 37°C).

The next objective of this study was to develop a method for the determination of the release profiles of the different NNs to evaluate their suitability as drug delivery devices for the controlled release of the protein. The NNs had to be cut in smaller pieces to ensure that every part releases comparable amounts of the protein at the same rate. This in turn led to the problem that the total amount of protein released per sample was rather low and the concentration was below the detection limit of UV/Vis spectroscopy. Thus we decided to

determine the amount of released protein via a BCA assay. Since this protein assay also gave rather low values, we validated it according to the ICH guidelines for the "Validation of analytical procedures". The experiments demonstrated that the BCA assay is a suitable detection method with a calculated detection limit of 1 µg/ml and a quantification limit of 3 µg/ml.

After successful validation of the BCA assay as suitable determination method, release profiles of the different NNs were determined. Depending on their solubility differently concentrated polymer solutions were prepared and 0.5 ml of an aqueous Cytochrome C solution was emulsified in 2 g of these polymer solutions. The resulting emulsions were eletrospun using appropriate electrospinning parameters. Protein loading and encapsulation efficiency strongly depended on the stability of the emulsion. PEC solutions formed the least stable emulsions with water. A part of the aqueous phase separated itself from the emulsion and was floating on the surface by the end of the elctrospinning procedure. As a consequence encapsulation efficiencies for PEC NNs were rather low at approximately 50%. Electrospinning emulsions for PLGA NNs were more stable and yielded encapsulation efficiencies of 80 to 85%. Emulsions consisting of a PLLA solution and an aqueous solution were the most stable with encapsulation efficiencies of 90 to 99%.

Figure 6 shows release profiles of the different NNs. PEC and PLGA NNs showed only a burst release and no further release of the protein. The amount of immediately released protein depended on the polymer concentration of the organic phase and was very high with values up to 50%. PLLA NNs showed no burst release and a rather slow prolonged released for the incorporated protein. The amount of released protein was almost identical for NNs prepared from 2% and 3% PLLA solutions. NNs electrospun from emulsions containing 1% PLLA showed a slightly increased release rate.

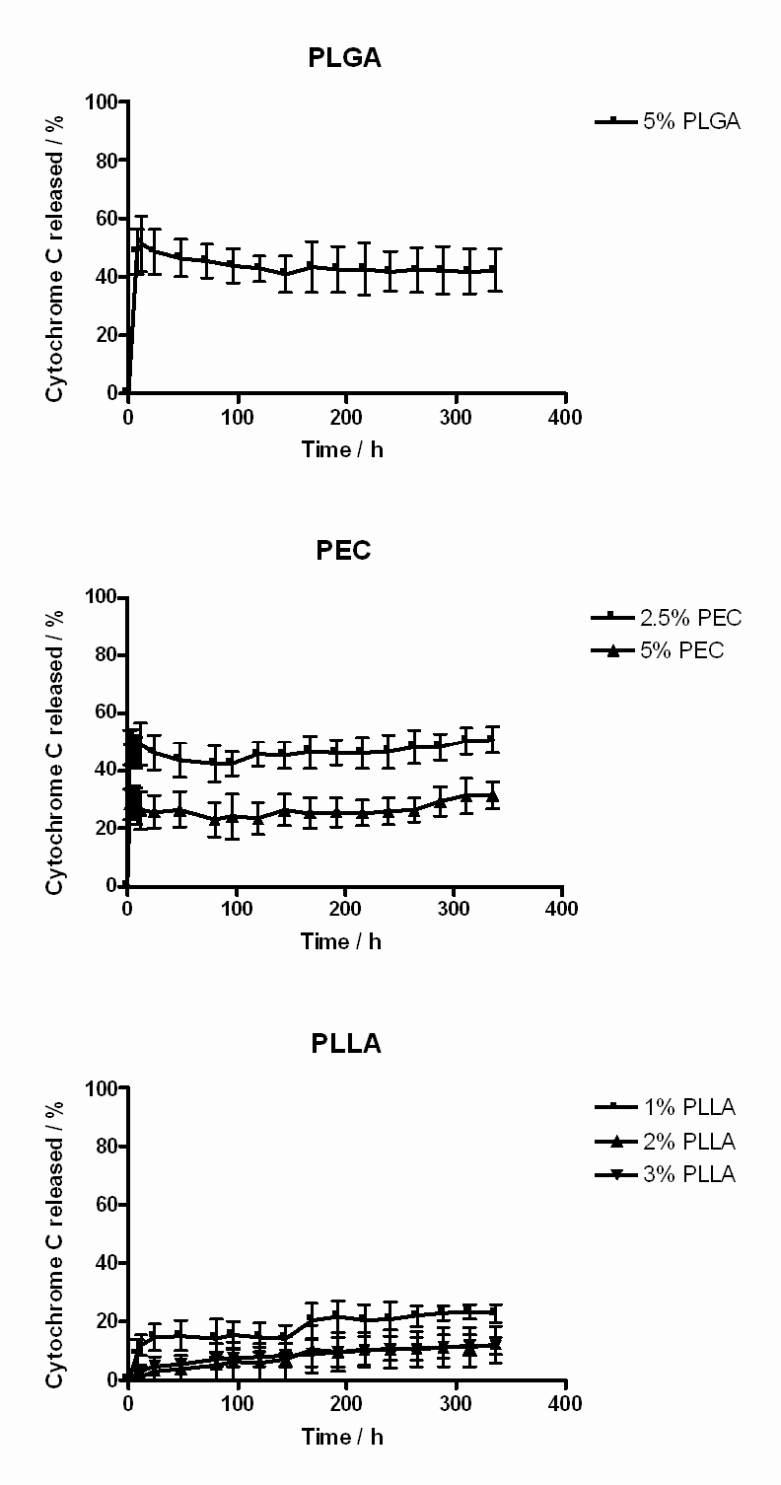

Figure 11: Protein release profiles of PLGA, PEC and PLLA nanofiber nonwovens.

At the end of the release study the NNs were withdrawn from the buffer solution and their morphologies were determined again using scanning electron microscopy. Figure 7 shows PEC NNs (a and b) and PLGA NNs (c and d) before and after the release study. Water uptake

led to swelling of the fibers and most fibers had lost their individuality and were more or less coalesced to a porous film especially in the case of PEC.

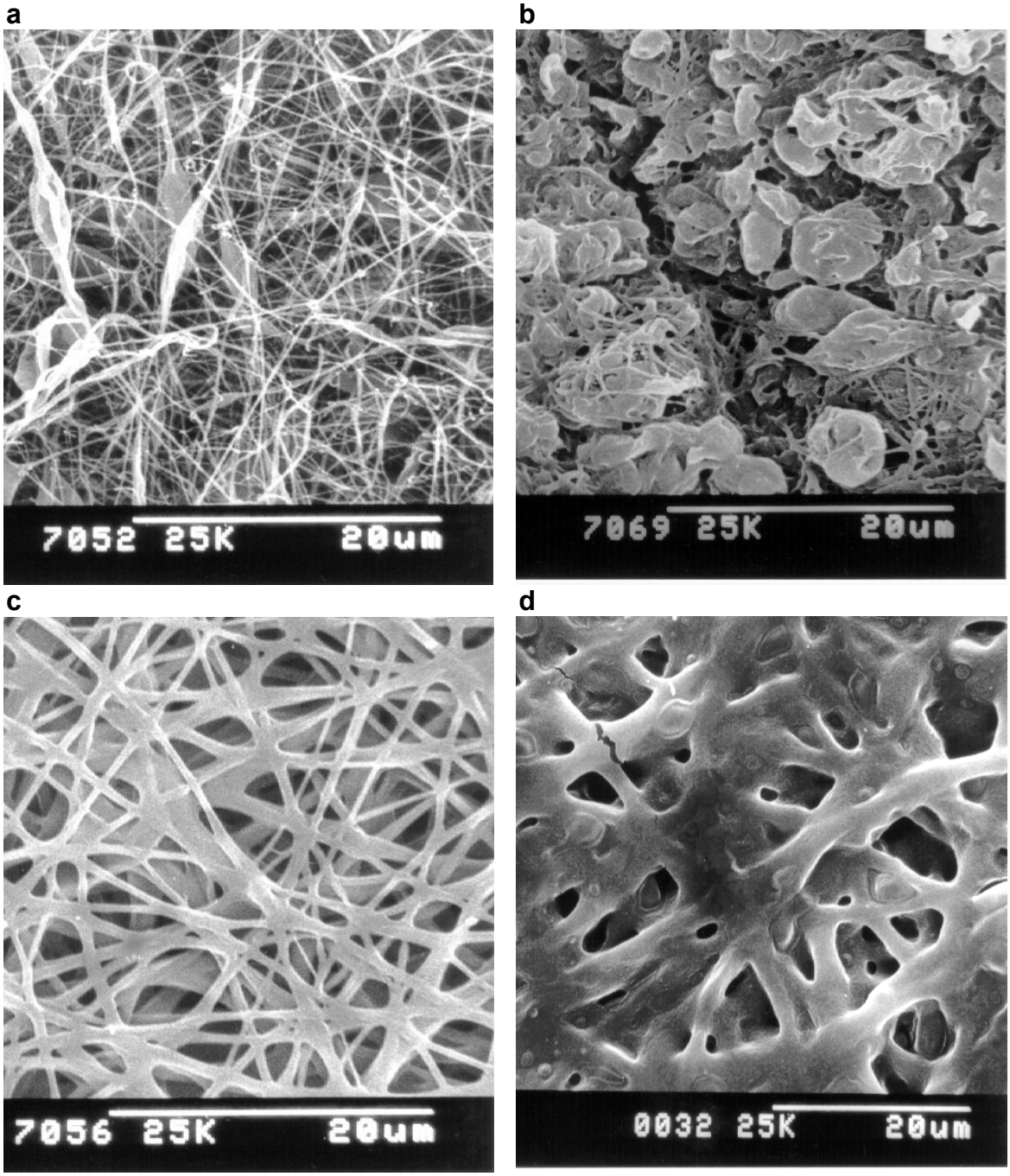

Figure 12: SEM images of nanofiber nonwovens (a: PLGA before release study, b: PLGA after release study; c: PLGA before release study, d: PLGA after release study)

PLLA fibers showed almost no sign of degradation or swelling at the end of the release study (Figure 8). Only at a low polymer concentration of 1% PLLA in the organic phase of the electrospinning emulsion, some fibers were coalesced.

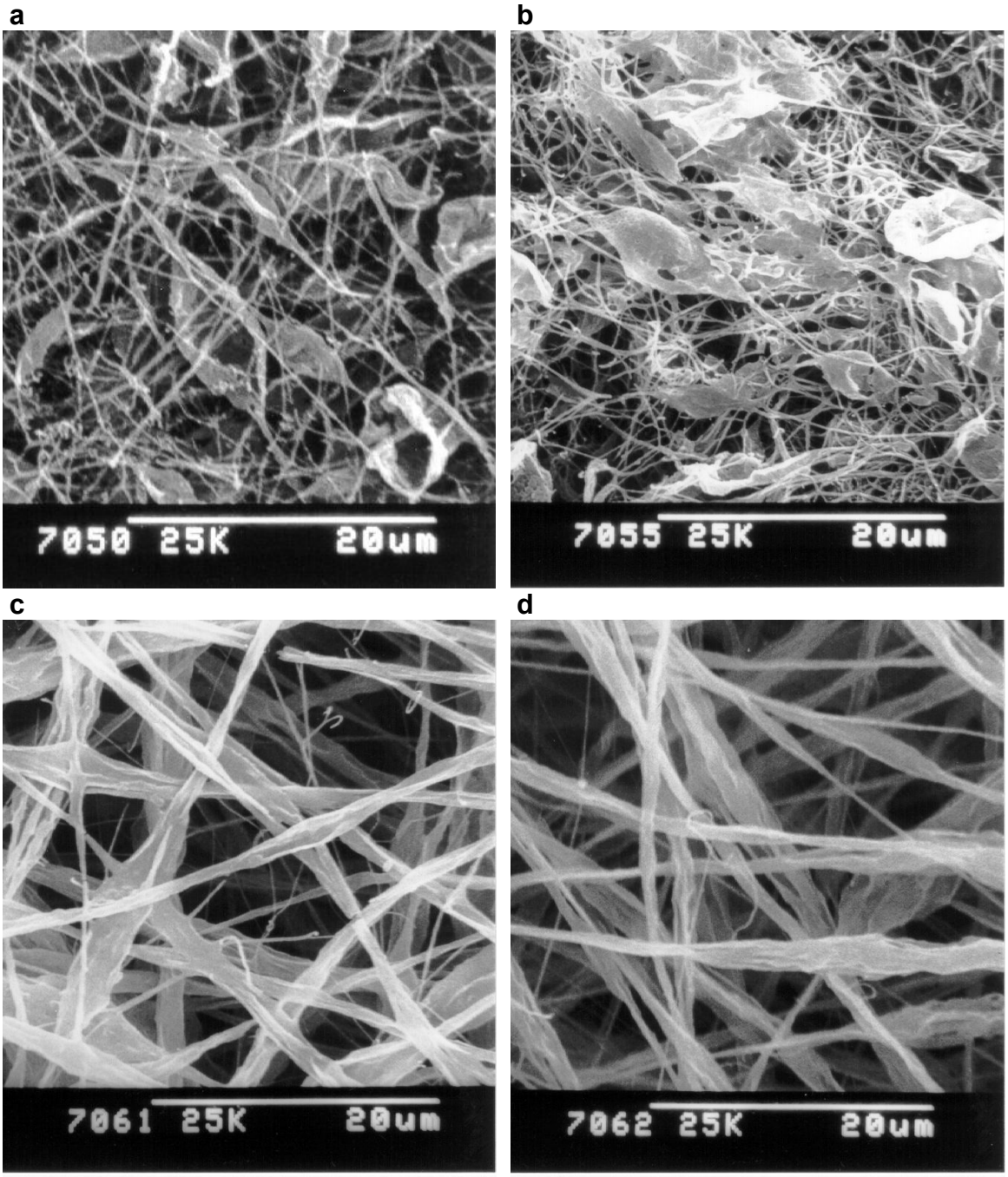

Figure 13: SEM images of nanofiber nonwovens (a: PLLA electrospun from 1% solution before release study, b: after release study; c: PLLA electrospun from 3% solution before release study, b: after release study)

In summary, PEC and PLGA NNs were not suitable for an application as protein delivery device, since they showed only a rather high burst release of the protein and no further release, which might be due to their morphological changes by swelling during the release study. PLLA NNs showed the most promising characteristics for their use as protein delivery devices. They are able to release the incorporated protein in a controlled manner for a longer period of time and due to their low swelling behavior their morphology was not affected during the release study.

Conclusion:

We demonstrated the feasibility to prepare protein loaded, biodegradable nanofiber nonwovens via electrospinning of emulsions. Suitable process parameters for the electrospinning of different polymers were successfully determined. PEC nanofiber nonwovens showed the most uniform fibers with the largest diameters of 1 – 5 µm. PLGA and PLLA fibers had diameters smaller than 1 µm depending on the polymer concentration in the organic phase. Lower polymer concentration also led to the formation of "burst bead" structures, which where formed by the formation of a polymeric skin on the liquid jet covering the droplets of the aqueous phase at first and subsequent collapsing. Native page gel electrophoresis proved that neither degradation nor aggregation of the protein occurred during electrospinning. Release studies demonstrated that only PLLA nanofiber nonwovens showed a prolonged release of the encapsulated protein. PEC and PLGA fibers showed high burst release of the protein and no further release within 2 weeks. SEM images demonstrated that PEC and PLGA fibers coalesced to porous films due to water uptake during incubation in buffer solutions. PLLA fibers showed almost no sign of degradation or swelling during the release study. In summary, nanofiber nonwovens composed of PLLA showed the highest potential as scaffolds for the controlled release of proteins.

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Chapter 3:

Electrospun biodegradable nanofiber nonwovens for controlled release of proteins

Published in *Journal of Controlled Release* (2008) 127(2): p. 180-187

Summary

Electrospinning of emulsions composed of an organic poly(L-lactide) solution and an aqueous protein solution yielded protein containing nanofiber nonwovens (NNs) having a mean fiber diameter of approximately 350 nm. Cytochrome C was chosen as a hydrophilic model protein compound for encapsulation. SEM imaging and gas adsorption measurements were carried out to determine morphology and surface characteristics of the different nanofiber nonwovens. Transmission electron microscopy was used to clarify the localization of the protein within the NN. PLLA NNs exhibited a highly hydrophobic surface which led to a slow wetting. It was shown that the protein release was dependent on the surface tension of the release medium. Electrospinning of emulsions consisting of an organic solution of PLLA and an aqueous solution of hydrophilic polymers yielded fibers composed of a polymer blend. The resulting NNs exhibited a less hydrophobic surface, which gave us the opportunity to tailor the release profile via this technology. Furthermore it was investigated how the addition of different amounts of hydrophilic polymer to the aqueous phase influenced the morphology of the resulting NNs.

Introduction

Polymeric drug delivery systems are able to improve therapeutic efficacy, reduce toxicity, and enhance compliance of the patients by delivering drugs at a controlled rate over a period of time to the site of action. In particular biodegradable polyesters and their copolymers demonstrated their potential as effective carriers for drug delivery [1, 2]. Electrospinning is a remarkably simple and powerful technique for the generation of polymeric fibers in the sub-micrometer scale, ranging from about 50 nm to several μm [3]. During the electrospinning process a polymer solution or melt is put through a capillary device to produce a droplet. This droplet of polymer solution is subject to a high electrical voltage (8 - 50 kV) which leads to electrostatic charging of the fluid and subsequently to formation of the so called Taylor cone [4]. At some point the electric force overcomes the surface tension of the droplet and a single fluid jet ejects at the apex of the Taylor cone. The jet accelerates in the electric field towards a counter electrode and undergoes a stretching and whipping process which leads to continuous elongation and solvent evaporation. Y.M. Shin et. al. investigated electrically forced jets in detail and came to the conclusion, that the rapidly whipping jet is a key step in the formation of submicron diameter fibers by electrospinning [5]. The polymeric fibers are collected on the counter electrode where they form a nonwoven fiber mat having a nano-structured surface.

Electrospinning is applicable to many types of polymer solutions or melts and represents an attractive approach for polymeric biomaterials processing [6-9]. The resulting nanofiber nonwovens are useful in a range of applications in different areas of research. They are particularly interesting for the field of biomedical engineering as wound dressings, scaffolds for tissue engineering, and drug delivery systems [10-17].

Encapsulation of low molecular weight drug substances like tetracycline hydrochloride [12], ibuprofen [18], rifampicin [8], and other molecules into electrospun NNs has already been demonstrated by a number of authors [19-24]. In most cases the drug was dissolved or

dispersed in the polymer solution and was encapsulated using a one phase electrospinning method. Only very few studies on the encapsulation and release of macromolecules like proteins have been published [9, 25-27]. Encapsulation of higher molecular weight substances is more difficult, because the electrospinning of macromolecular dispersions is more challenging than electrospinning of homogenous polymer solutions. The concept of coaxial electrospinning led to core-shell structured fibers which proved their suitability as drug delivery devices for larger molecules, but special equipment is needed for these electrospinning techniques [28-30]. Another approach which has not been investigated very extensively until now is the electrospinning of emulsions [26, 27, 31, 32].

In this study we present the preparation of highly hydrophobic, protein loaded nanofiber nonwovens (NNs) based on poly(L-lactic acid) (PLLA) via electrospinning of emulsions and their characterization by SEM and gas adsorption measurements. Cytochrome C was chosen as a hydrophilic model protein due to the ease of detection and quantification methods available for this compound. The release of macromolecules was shown to be affected by the hydrophobicity of the NNs, and therefore, we were able to modify the release profiles by blending the PLLA with hydrophilic polymers such as poly(ethylene imine) (PEI) and poly(L-lysine) (PLL).

Materials and methods:

Poly(L-lactide) (PLLA) Resomer L210 (inherent viscosity 3.6 dl/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(ethylene imine) (25 kDa, PEI) was a gift from BASF, Ludwigshafen, Germany. Poly(L-lysin) (PLL) was purchased from Sigma Chemical Co. (St. Louis, USA). Cytochrome C from horse heart was obtained from Fluka Chemie GmbH (Buchs, Germany). All other reagents used were of analytical grade.

Electrospinning

Different emulsions were prepared in 5 ml syringes using an Ultra Turrax T25 running at 20500 rpm for 1 minute. A setup published previously was used for electrospinning [33]. Electrospinning was carried out using a variable high-voltage power supply, which can produce voltages ranging from 0 to 55 kV. It consists of one positive power supply attached to a blunt steel needle and one negative power supply attached to the counter electrode. The syringes containing the different emulsion were attached to the blunt needle and were put into a syringe pump pushing 50 µl of the emulsions per minute through the blunt needle. A round steel plate with a diameter of 9 cm served as the counter electrode. It was covered with aluminum foil for the collection of the different fibers.

PLLA nanofiber nonwovens were prepared by emulsifying 0.5 ml of an aqueous solution of Cytochrome C (20 mg/ml) into 2 g of 1, 2, or 3 wt% PLLA in chloroform. The viscosity of an emulsion containing 2 wt% PLLA was 921 mPa · s. A voltage of +19 kV was applied at the needle tip, and –1 kV was applied at the counter electrode, which was 20 cm from the needle tip. In the case of PLLA / PEI and PLLA / PLL blends, the nanofiber nonwovens were prepared by dissolving either PEI or PLL in the aqueous phase before the emulsification into the chloroform containing PLLA. The content of PEI or PLL in the blends is presented as the wt% relative to the mass of PLLA. When electrospinning these blends, the applied voltage values were +20 kV at the needle tip and -2 kV at the counter electrode, with the distance maintained at 20 cm.

SEM imaging

Fragments of the nanofiber nonwovens were sputtered with gold in an argon atmosphere (0.3 mPa) using a sputter coater S-150 (Edwards Kniese & Co. GmbH, Marburg, Germany). Sputtering was carried out for each sample at 18 mA 2 times for 1 minute and after that 2

times for 2 minutes. Images were taken with an SEM S-510 at 25 kV accelerating voltage (Hitachi Ltd. Corporation, Tokyo, Japan) using the Digital Imaging Scanning System (Version 5.4.12.1) purchased from point electronic GmbH, Halle (Saale), Germany. Images were analyzed with the Digital Image Processing System (Version 2.6.13.0) purchased from point electronic GmbH, Halle (Saale), Germany.

TEM imaging

Fragments of the nanofiber nonwovens were directly imaged by transmission electron microscopy (TEM) using a JEM 3010 operated at 300 kV without staining or cutting of the samples. Images were created by Digital Micrograph Software (Gatan Inc.).

Contact angle

Static contact angles of water on the nanofiber nonwoven surface were measured using the sessile drop method with a G10 Drop Shape Analysis System (Krüss GmbH, Hamburg, Germany). 5 µl deionized water were dropped onto a dry nanofiber nonwoven in air and the contact angle was calculated using Data Physics SCA20 Contact Angle Analyzer Software.

Gas adsorption measurements

Gas adsorption measurements were carried out using a BELSORP-mini (BEL Japan, Inc.) in high precision mode. In this mode the saturated vapor pressure and dead volume are corrected from the actual measurement. BELSORP-mini uses a volumetric gas adsorption method. Samples were pretreated using a heater for 24h at 37°C by vacuuming. Dead volume measurements were carried out at room temperature using helium gas. Adsorption and desorption measurements were performed with the sample cell and an empty reference cell immersed in liquid nitrogen to maintain a constant temperature. Dead volume changes due to

evaporation of liquid nitrogen. Therefore the dead volume of the reference cell was measured prior to every adsorption measurement. Nitrogen gas was used as adsorbent.

Loading

Loading of the nanofiber nonwovens was determined by degrading parts of the nanofiber nonwovens in 1 ml of 1 N NaOH. The amount of incorporated Cytochrome C was calculating by measuring the adsorption of the resulting solution at 412 nm using a UV/Vis spectrophotometer UV-160 (Shimadzu Corporation, Kyoto).

Release profile

Release studies were carried out in PBS buffer solution pH 7.4. Samples of the nanofiber nonwovens (2 cm x 2 cm) were put in 15 ml plastic tubes and 2 ml release medium was added. The tubes were constantly moved at a speed of 30 rpm at 37°C. At certain time points 200 µl of the release medium were removed and replaced with fresh buffer solution. The amount of released cytochrome C was determined via BCA assay. 50 µl of the samples were mixed with 170 µl BCA reagent (BCA assay kit, Pierce, Rockford IL, USA) in 96 well plates. After 18h at ambient temperature the absorption at 560 nm was measured using a plate reader. All measurements were carried out in triplicate.

Results and Discussion:

1. *Preparation and characterization of nanofiber nonwovens*

At first the influence of the polymer concentration on the morphology of the resulting fibers was examined. Five different emulsions with a concentration ranging from 1 – 3 wt% poly(L-lactic acid) in the organic phase were electrospun. With increasing polymer concentration the uniformity of the fibers and the mean fiber diameter increased (Fig.1 a-c).

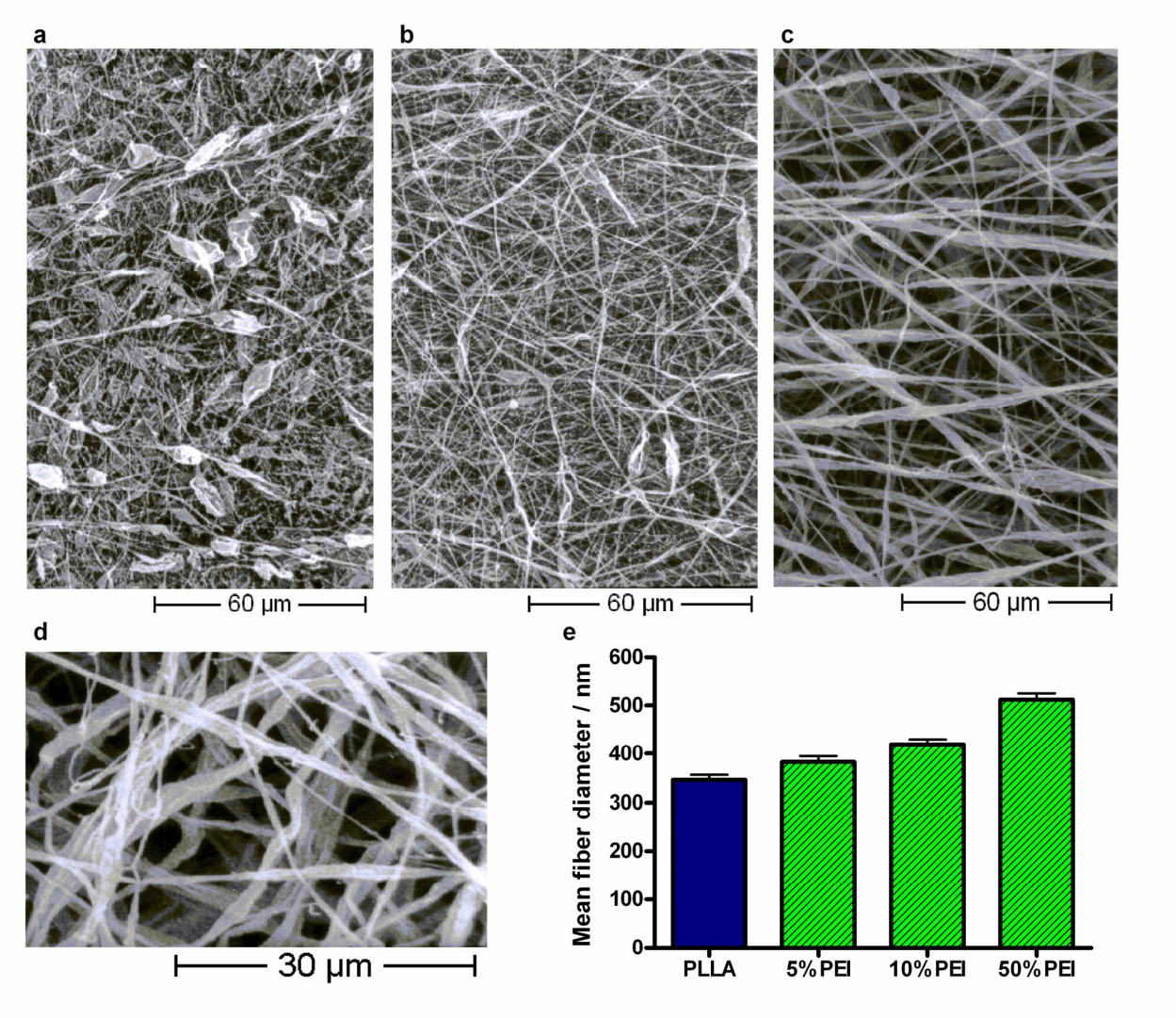

Figure 14: SEM images of NNs with incorporated cytochrome C electrospun from emulsions having differently concentrated organic phases (a: 1% PLLA, b: 2% PLLA, c: 3% PLLA). NNs show different morphology depending on the polymer concentration. d: NN blended with 50% PEI shows comparable morphology to pure PLLA NNs. Addition of hydrophilic polymers leads to an significant increase in mean fiber diameter depending on the amount of added polymer (e).

Especially at lower polymer concentrations structures that looked like burst beads were observed. These structures are most likely due to the formation of a polymeric skin on the liquid jet covering the droplets of the aqueous phase at first and then collapsing. Other groups have already reported similar fiber morphology even using one-phase electrospinning techniques [34-36]. As a consequence of the collapsing beads, not the whole amount of cytochrome C is encapsulated in the nanofibers. TEM images revealed crystallite structures having a size of 200 – 500 nm outside the fibers which are most likely composed of

cytochrome C (Figure 2). Nevertheless those protein crystals were still encapsulated within the nonwoven fiber mat.

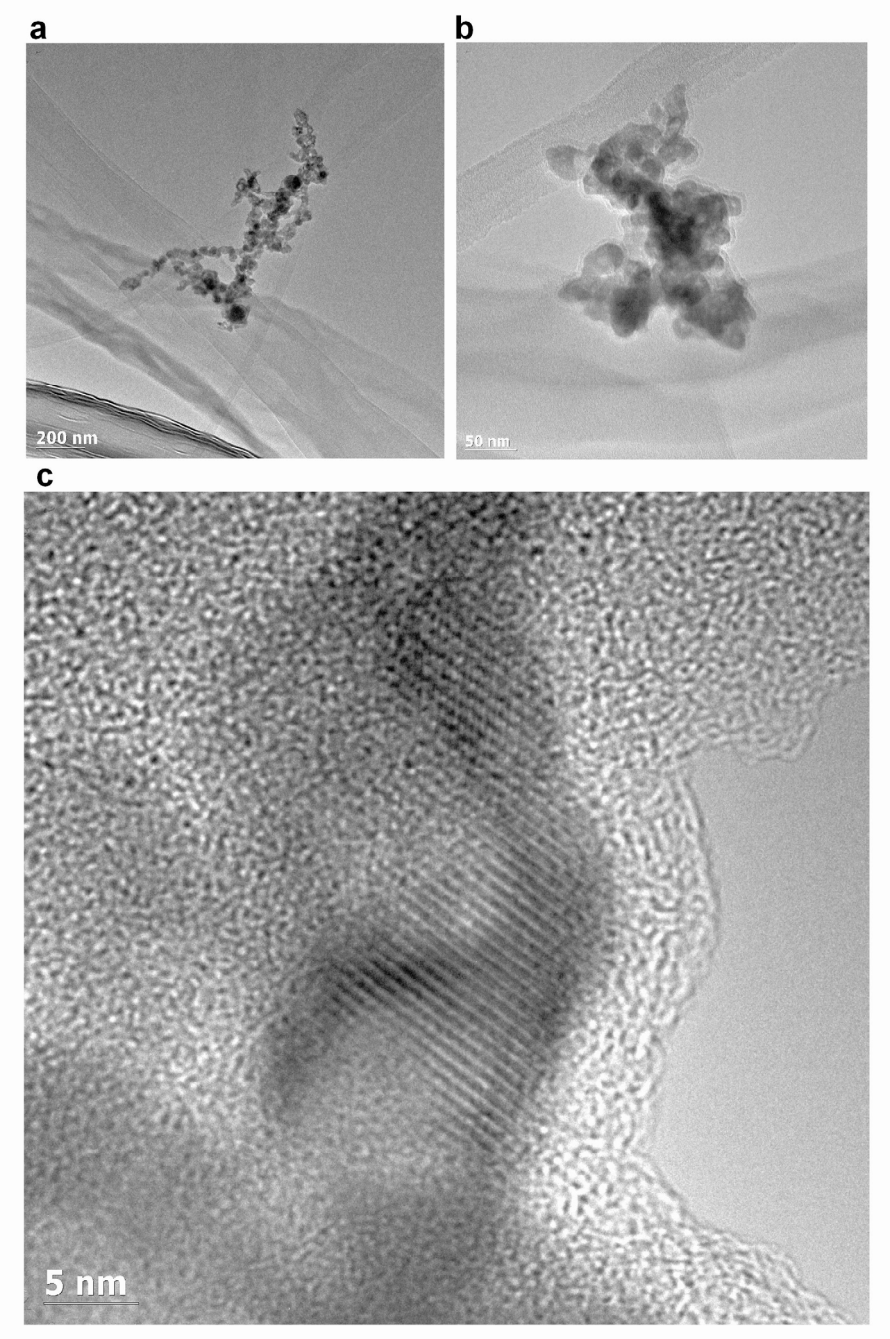

Figure 15: TEM images of NNs with incorporated cytochrome C electrospun from emulsions showing crystallite structures outside the fibers (a, b: examples of crystallite structures; c: magnification of figur 2b showing a periodic pattern, which is characteristic for crystallite structures). Samples were put on a supporting grid and directly imaged without any staining or embedding of the samples.

Static contact angles of water on the NN surface were determined using a sessile drop method. No significant differences between NNs prepared from differently concentrated

polymer solutions were observed and the mean contact angle was 109.4 ± 5.9 ° ($p < 0.05$). The high contact angle of water on PLLA NNs shows their superhydrophobicity, which is the reason for the very slow wetting of these devices. The hydrophobicity of the PLLA NNs was reduced by addition of hydrophilic polymers like poly(ethylene imine) or poly(L-lysine) to the aqueous phase of the electrospinning emulsion. We chose charged hydrophilic polymers since they also increase the conductivity of our electrospinning emulsion. A higher conductivity facilitates electrospinning of these emulsions. The morphology of the resulting polymer blend fibers was similar to pure PLLA fibers. Even with an addition of 50 % PEI there were no differences in the macroscopic fiber morphology visible on SEM images (Fig. 1d). After analysis of the SEM images using the measuring tool of the "Digital Image Processing System" software it could be demonstrated that the mean fiber diameter increases significantly after addition of hydrophilic polymers with regard to the amount of added polymer (Fig 1e). Fibers electrospun from emulsions containing 2 wt% PLLA showed a mean fiber diameter of 346.6 ± 10.1 nm. The addition of 5% PEI to the aqueous phase of the emulsion led to a significant increase in mean fiber diameter to 383.4 ± 12.0 nm ($p < 0.05$). Further increases in PEI content in the blend fibers led to further increases in fiber diameter, with mean fiber diameters of 419.1 ± 10.8 nm at 10% PEI and 511.8 ± 13.1 nm at 50% PEI. It is expected that the addition of polyelectrolytes would increase charge density and decrease the fiber diameter [37]. In this case this effect is overcome by the increasing polymer concentration in the emulsion, which leads to an increase in mean fiber diameter.

Native page gel electrophoresis was used to check whether the electrospinning process would have a negative effect on the protein. There were no visible changes in retention time between native cytochrome C and the cytochrome C released from the NNs. These results prove that neither degradation nor aggregation of the protein occurred during electrospinning. Other groups have also shown that electrospun proteins are stable and retain their bioactivity [9, 25].

Usually NNs are characterized just by calculating their mean fiber diameter from SEM images. But since not just single nanofibers are used, but a nonwoven consisting of those nanofibers, we also wanted to examine the device as a whole. Gas adsorption measurements were carried out to characterize the surface properties of the NNs in detail. A volumetric method was used to determine the amount of adsorbed gas. After evacuation of the sample cell a specified amount of nitrogen gas was added to the sample cell. The pressure of adsorption gas in the sample cell decreases as adsorption occurs. The adsorption amount was measured by calculating the differences between pressures before and after gas adsorption or desorption respectively. To assure reproducibility of the experiment, five PLLA NNs that were prepared from 5 different emulsions having the same composition were characterized.

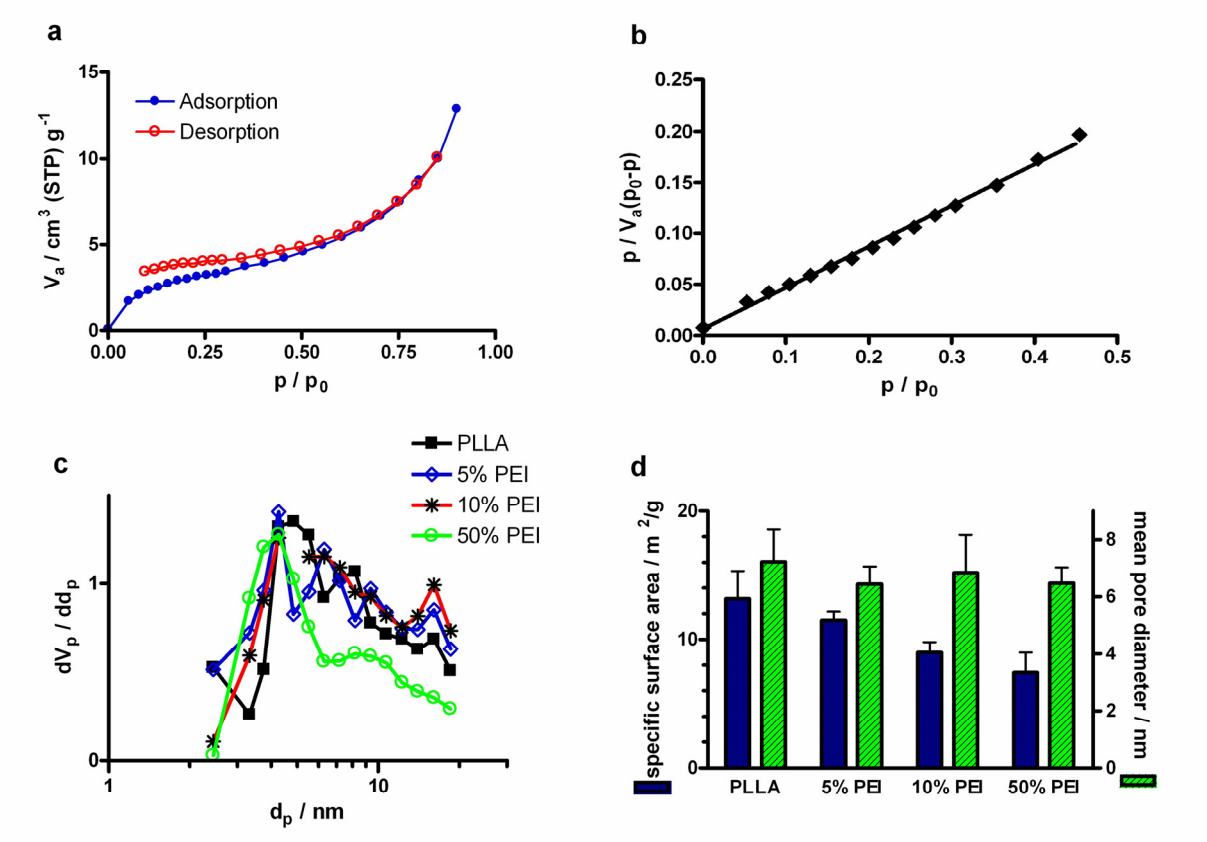

Figure 16: a: Gas adsorption and desorption isotherm of a PLLA NN electrospun from an emulsion containing 2% PLLA in the organic phase. b: BET plot of the PLLA NN which is used to calculate the specific surface area and mean pore diameter of the NN. c: Pore size distribution of different NNs is given in the BJH plot (PLLA: filled squares; PLLA/PEI 5%: open squares, PLLA/PEI 10%: stars, PLLA/PEI 50%: open circles). d: Blending of PLLA NNs leads to an decrease of the specific surface area. The mean pore diameter is not affected.

The resulting adsorption and desorption isotherms were highly reproducible for one NN and very similar in comparison to the other PLLA NNs, even though the amount of adsorbed gas was rather low. Figure 3a shows an adsorption and desorption isotherm of a PLLA NN electrospun from an emulsion containing 2 wt% PLLA in the organic phase. Adsorption isotherms are classified based on the strength of the interaction between the sample surface and adsorptive gas, and the existence or absence of pores. The desorption isotherms of the examined PLLA NNs show hysteresis compared to the adsorption isotherms. This effect can be explained by the presence of mesopores with a diameter of 2 – 50 nm on the surface of the fibers. Hysteresis occurs because capillary condensation of nitrogen gas takes place in mesopores and there is a difference in meniscus during adsorption and desorption. The monolayer volume of adsorbed gas (V_m) was determined according to the BET theory. The BET plot (Figure 3b) should give a linear line in a relative pressure range from 0.05 to 0.35 and the slope of that line is used to determine V_m, from which we can calculate the specific surface area of the NNs. In this manner we determined a specific surface area of 13.17 $m^2/g \pm$ 2.15 for the PLLA NNs. NNs composed of nanofibers consisting of a polymer blend of PLLA and poly(ethylene imine) showed a decreased specific surface area with regard to the amount of added hydrophilic polymer (Figure 3d). An addition of 5% poly(ethylene imine) to the aqueous phase of the electrospinning emulsion does not decrease the specific surface area of the resulting NNs significantly (11.49 ± 0.70 m^2/g), but the addition of 10% or 50% PEI led to a significantly decreased specific surface area (8.97 ± 0.82 m^2/g for PLLA/PEI 10% and 7.43 ± 1.54 m^2/g for PLLA/PEI 50%; $p < 0.05$). This effect is caused by the increased mean fiber diameter of the NNs composed of fibers consisting of a polymer blend. As demonstrated via SEM imaging, the mean fiber diameter increases significantly with increasing amount of hydrophilic polymer. This increase in mean fiber diameter will naturally result in a decrease in specific surface area. Considering the density of the adsorbent at 77 K it is possible to

specify the mean pore diameter of the sample. In this manner a mean pore diameter of 7.22 ± 1.14 nm was calculated for the surface pores on PLLA fibers. Blending with PEI did not lead to significant differences in mean pore diameter ($p < 0.05$). The pore size distribution was determined from the desorption isotherm according to [38]. The so called BJH plot is expressed as percentage change of pore volume ($\Delta V_p/\Delta r_p$) against mesopore diameter. BJH-plots of all fibers showed a monomodal distribution of mesopores matching the calculated size from the BET plot (Figure 3c). All distributions showed a peak at a diameter of roughly 4.5 nm. The addition of PEI did not have an influence on pore size distribution.

2. Protein release characteristics of nanofiber nonwovens

Loading of the NNs has been determined after hydrolytic degradation of the polymer in basic solution. They showed high encapsulation efficiencies of 85% to 95%. Loading of each NN was determined prior to the release studies to calculate the release percentage of the actual protein content of every single NN.

At first the influence of the polymer concentration in the organic phase on the release profile of PLLA NNs was investigated. For this purpose PLLA NNs electrospun from 1 wt%, 2 wt% or 3 wt% PLLA solution were immersed in PBS buffer solutions and the amount of released protein was determined via BCA assays. PLLA NNs electrospun from 2 wt% and 3 wt% solutions showed no burst release, implying the perfect inclusion of the protein within the NN. NNs prepared from emulsions containing only 1 wt% PLLA in the organic phase show a slightly increased release. Only very slow release of the incorporated protein can be observed in the following 30 days (Figure 4). Some studies about drug release from NNs have already been published. To clarify the release mechanism from NNs it is important to know where the drug is localized inside the NN. The encapsulation of lipophilic compounds usually leads to the drug being homogenously dispersed inside the fibers.

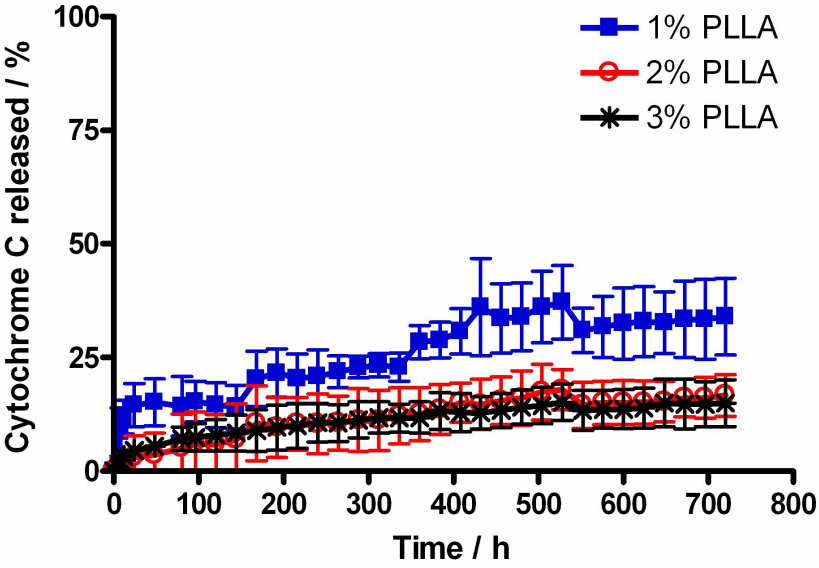

Figure 17: Release profiles of cytochrome C from PLLA NNs electrospun from emulsions having differently concentrated polymer solutions as organic phase.

J. Zeng et. al. [8] encapsulated rifampicin in PLLA fibers using a one phase electrospinning technique. They observed a release of the drug only in the presence of the enzyme proteinase K which degrades the PLLA fibers. On the other hand hydrophilic compounds are usually located on the surface of the fibers. K. Kim et. al. [21] applied an one phase encapsulation technique for the encapsulation of the hydrophilic antibiotic cefoxitim sodium in poly(lactide-co-glycolide) NNs. They observed a very high burst release due to limited physical interactions between drug and polymer matrix. We showed for our NNs, that Cytochrome C exists in crystallite form in between the fibers of the NNs (figure 2). Considering these findings one would expect at least some burst release for our hydrophilic model protein cytochrome C. The reason for the very slow release from the NNs described in this study is their very slow wetting as a consequence of the highly hydrophobic surface characteristics. The slightly increased release for NNs electrospun from 1 wt% solutions can be addressed to the decreased thickness of the NNs which leads to better wetting behavior. To clarify the effect of wettability on the protein release, another set of release studies was carried out using

release media having different surface tensions through the addition of an emulsifier. Prior to the release studies, the CMC of the emulsifier Tween 20 in PBS buffer solution was determined. After that 6 buffer solutions were prepared having emulsifier concentrations below the CMC, at the CMC, and above the CMC. Addition of an emulsifier to the release medium increased the protein release. Buffer solutions having an emulsifier concentration at the CMC or above the CMC led to complete release of the protein within a few hours. For concentrations below the CMC we determined a surface tension related release of the protein (Figure 5).

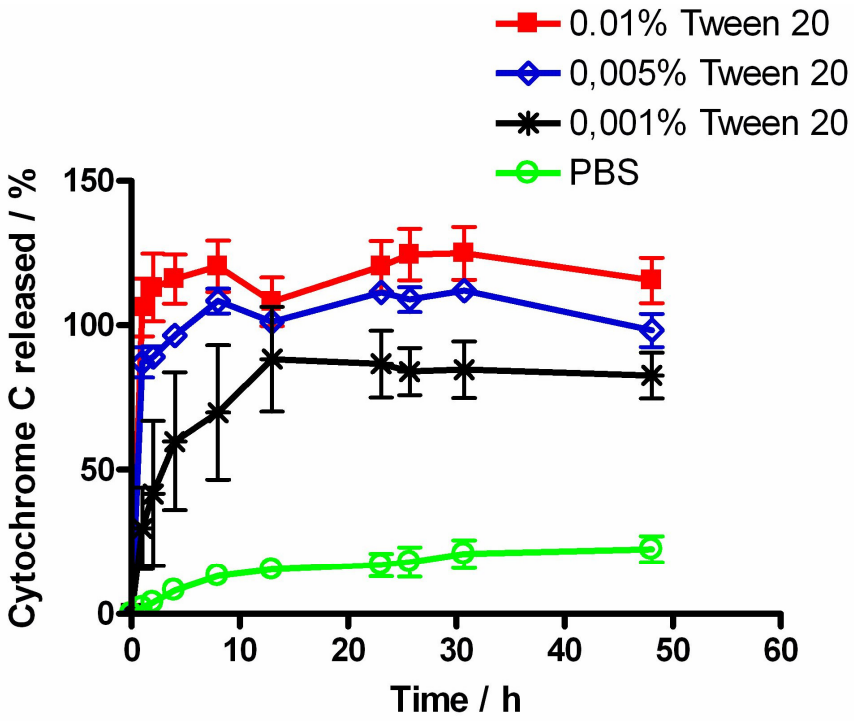

Figure 18: Release profiles of PLLA NNs with incorporated cytochrome C determined in buffer solutions having different surface tensions (all concentrations of the emulsifier are below the CMC).

Higher concentrations of Tween 20 led to a faster release of the incorporated protein. This proved that the wettability of the NNs is predominantly responsible for the release rate and that the major part of cytochrome C was encapsulated within the NN but not within the fibers itself. These results gave us the opportunity to tailor the release profile of the NNs by adjusting their hydrophobicity. Thus, we decided to produce NNs with less hydrophobic

surfaces via the addition of hydrophilic polymers to the aqueous phase of the electrospinning emulsion. NNs composed of these polymer blend fibers should show a faster protein release compared to pure PLLA NNs. Blending with hydrophilic polymers has already been applied by other groups to decrease the burst release of hydrophilic drug substances [18, 21]. In contrast to their approach our intention was to increase and control the drug release. For that reason aqueous poly(ethylene imine) 25 kDa (PEI) and poly(L-lysine) solutions were prepared. Cytochrome C was dissolved in these polymer solutions and the resulting solution was used as the aqueous phase of the electrospinning emulsions. The resulting NNs showed release profiles depending on the amount of added hydrophilic polymer (Figure 6). With increasing amount of hydrophilic polymers the hydrophobicity of the NNs was reduced and the protein release rate was increased. Blending of the NNs influenced primarily the initial release phase and resulted in the opportunity to tailor the release profile of the NNs.

In summary, we present an electrospinning technique that allows the encapsulation of hydrophilic proteins in biodegradable NNs which do not show the common but unfavorable high burst release. Although it is known that electrospun NNs may have super-hydrophobic surfaces [39], this study showed the importance of that feature for drug release in detail and how it is possible to exploit this potential to adjust the release profile of these drug delivery devices.

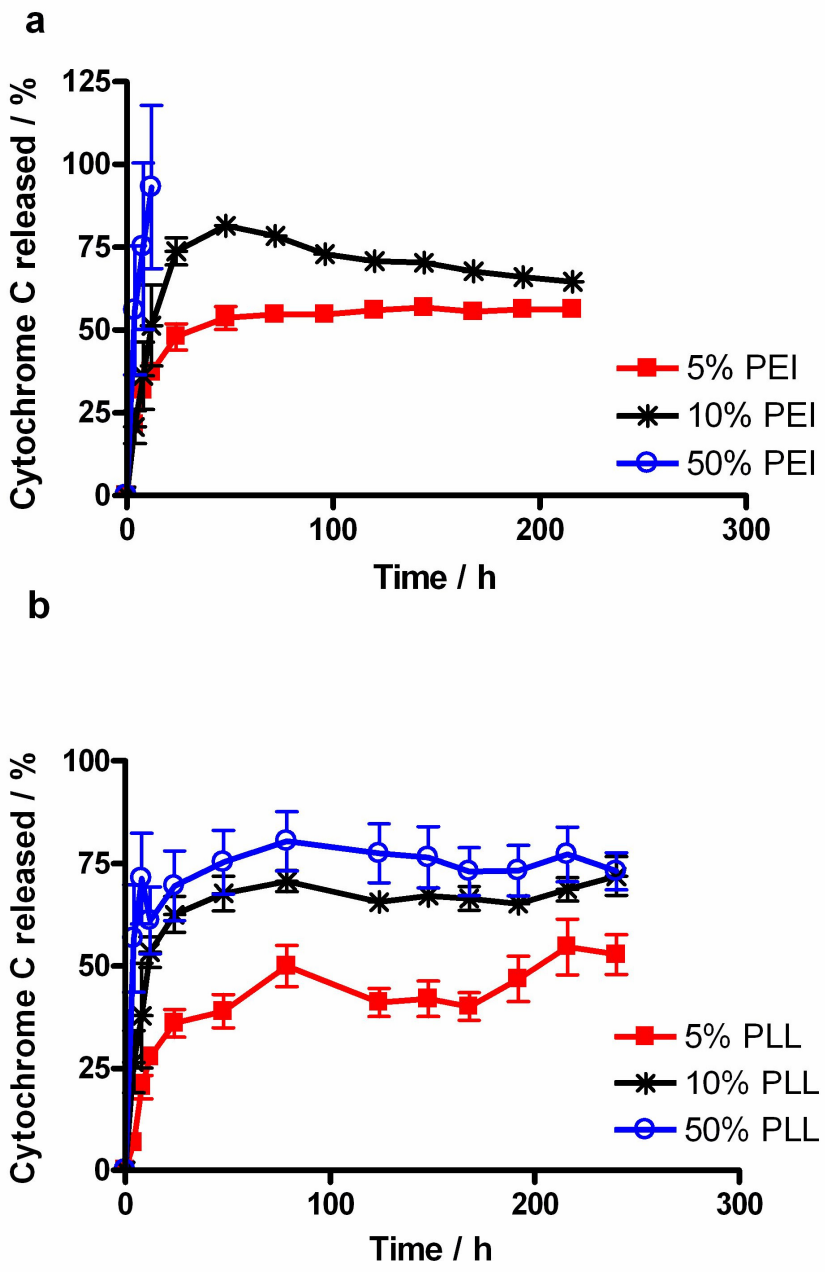

Figure 19: Release profiles of PLLA NNs blended with different amounts of hydrophilic polymers (a: blended with PEI, b: blended with PLL).

Conclusion:

We showed the feasibility to prepare protein loaded nanofiber nonwovens via electrospinning of emulsions consisting of an organic polymer solution and an aqueous protein solution. SEM images demonstrated that the mean fiber diameter is in a nanometer scale and increases with increasing polymer concentration. The addition of hydrophilic polymers to the aqueous phase

of the electrospinning emulsion yielded NNs composed of nanofibers consisting of a polymer blend. Mean fiber diameters of these NNs increased with increasing amounts of hydrophilic polymer in the aqueous phase. Gas adsorption measurements were carried out to investigate surface properties in detail like specific surface area, mean pore size, and pore size distribution. We demonstrated that the increased fiber diameter of the NNs composed of polymer blend nanofibers led to a decrease of their specific surface area. PLLA NNs exhibited a highly hydrophobic surface, which also proved to be the major criterion controlling the protein release. The release rate of PLLA NNs was rather slow, but NNs composed of nanofibers consisting of a polymer blend of PLLA and a hydrophilic polymer exhibited adjustable protein release rates depending on the amount of added hydrophilic polymer.

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Chapter 4:

Protein loaded electrospun nanofiber nonwovens based on poly (L-lactide) as biodegradable scaffolds for tissue engineering

Summary

A key requirement for tissue engineering is the design of a scaffold which exhibits suitable biological and physical properties. The incorporation of bioactive substances, which are essential for cell growth or differentiation, would also be very advantageous. In this study protein loaded nanofiber nonwovens (NNs) were prepared by electrospinning of emulsions to investigate their suitability as tissue engineering scaffold. The hydrophobicity of the resulting NNs was adjusted by the addition of hydrophilic polymers to the aqueous phase of the electrospinning emulsion. Five different NNs based on PLLA and blends thereof were investigated for their suitability as scaffolds for tissue engineering. The first part of this study demonstrated that NNs showed a mean fiber diameter of 350 – 500 nm, which is similar to the fibrous protein structures of the extracellular matrix (ECM). Cell adhesion, cell viability and cell morphology on different NNs were determined and compared to a glass surface. The results of this study demonstrated that there were no significant differences for cells grown on NNs compared to a glass surface. NNs composed of a blend of 95% PLLA and 5% PLL featured the most promising attributes for their use as tissue engineering scaffolds, since they showed significantly higher values for cell adhesion and the best cell spreading behaviour of all NNs.

Introduction

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue functions or a whole organ [1]. The creation of new tissues under in vitro conditions is based on three principles: the isolation and cultivation of cells, the use of tissue inducing substances and the placement of cells within suitable matrices or on polymeric scaffolds. The preparation of a scaffold which provides suitable biological and physical properties is a critical requirement for tissue engineering. Every tissue can be described as a microenvironment which is composed of parenchymal cells (functional cells) and mesenchymal cells (support cells) contained within an extracellular matrix (ECM). Therefore a suitable tissue engineering scaffold should mimic the nanofibrous features of the ECM. The scaffold material needs to be biocompatible, should promote cell adhesion and proliferation and in particular should support their three dimensional growth. Finally the scaffold should be biodegradable in order that the artificial matrix can be replaced by a new natural ECM after the differentiation of the seeded cells to viable tissue.

The ECM in native tissues consists of fibrous proteins with a diameter of 50 – 500 nm. Electrospinning is a unique technology for the generation of polymeric fibers in the sub-micrometer scale, ranging from about 50 nm to several μm [2]. This simple and versatile technique is applicable to many types of polymer solutions or melts and represents an attractive approach for the production of tissue engineering scaffolds with structures very similar to the ECM [3-8]. Furthermore, electrospinning of emulsions allows bioactive substances to be incorporated in these electrospun nanofiber nonwovens. These substances will be released from the fibers in a controlled manner for example to support cell growth or induce differentiation. Hence these scaffolds act not only as cell carriers but also as controlled release systems.

Standard electrospinning instrumentation usually consists of three major components: a high voltage power supply, a spinneret (a metal capillary, e.g. a needle with blunt tip) and a grounded collecting device (a metal plate or a rotating drum). During the electrospinning process a polymer solution or melt is pushed through the spinneret to produce a droplet. This droplet is subject to a high electrical voltage (8 - 50 kV) which leads to electrostatic charging of the fluid and subsequently to formation of the so called Taylor cone [9]. At some point the electric force overcomes the surface tension of the droplet and a single fluid jet ejects at the apex of the Taylor cone. The jet accelerates in the electric field towards a counter electrode and undergoes a stretching and whipping process which leads to continuous elongation and solvent evaporation [10]. The polymeric nanofibers are collected on the counter electrode where they form a nonwoven fiber mat.

The objective of this study was to investigate the suitability of NNs based on PLLA for their application as tissue engineering scaffolds. The NNs were prepared via electrospinning of emulsions. The aqueous phase contained cytochrome C as a model substance for the incorporation of growth or differentiation factors which will be released from the NNs in a controlled manner. Cell adhesion, cell viability and cell morphology on these scaffolds composed of PLLA or blends of PLLA and hydrophilic polymers was determined and the impact of the addition of hydrophilic polymers was investigated.

Materials and methods

Materials

Poly(L-lactide) (PLLA) Resomer L210 (inherent viscosity 3.6 dl/g) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(ethylene imine) (25 kDa, PEI) was a gift from BASF, Ludwigshafen, Germany. Poly(L-lysine) (PLL) was purchased from Sigma

Chemical Co. (St. Louis, USA). Cytochrome C from horse heart was obtained from Fluka Chemie GmbH (Buchs, Germany). All other reagents used were of analytical grade.

Cell culture

L-929 (mouse subcutaneous connective tissue; ATCC: CCL-1) cells were purchased from ATCC (Teddington, UK) and maintained in DMEM (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal calf serum (Cytogen, Sinn, Germany) in humidified atmosphere with 5% CO_2 at 37 °C.

Preparation of nanofiber nonwovens

Different emulsions were prepared in 5 ml syringes using an Ultra Turrax T25 running at 20500 rpm for 1 minute. A setup published previously was used for electrospinning [11]. Electrospinning was carried out using a variable high-voltage power supply, which can produce voltages ranging from 0 to 55 kV. It consists of one positive power supply attached to a blunt steel needle and one negative power supply attached to the counter electrode. The syringes containing the different emulsion were attached to the blunt needle and were put into a syringe pump pushing 50 µl of the emulsions per minute through the blunt needle. A round steel plate with a diameter of 9 cm served as the counter electrode. It was covered with aluminium foil and round glass cover slips were used to collect the different fibers.

PLLA nanofiber nonwovens were prepared by emulsifying 0.5 ml of an aqueous solution of Cytochrome C (20 mg/ml) into 2 g of 1, 2, or 3 wt% PLLA in chloroform. The viscosity of an emulsion containing 2 wt% PLLA was 921 mPa · s. A voltage of +19 kV was applied at the needle tip, and –1 kV was applied at the counter electrode, which was 20 cm from the needle tip. In the case of PLLA / PEI and PLLA / PLL blends, the nanofiber nonwovens were prepared by dissolving either PEI or PLL in the aqueous phase before the emulsification into

the chloroform containing PLLA. The content of PEI or PLL in the blends is presented as the wt% relative to the mass of PLLA. When electrospinning these blends, the applied voltage values were +20 kV at the needle tip and -2 kV at the counter electrode, with the distance maintained at 20 cm.

SEM imaging

Fragments of the nanofiber nonwovens were sputtered with gold in an argon atmosphere (0.3 mPa) using a sputter coater S-150 (Edwards Kniese & Co. GmbH, Marburg, Germany). Sputtering was carried out for each sample at 18 mA 2 times for 1 minute and after that 2 times for 2 minutes. Images were taken with an SEM S-510 at 25 kV accelerating voltage (Hitachi Ltd. Corporation, Tokyo, Japan) using the Digital Imaging Scanning System (Version 5.4.12.1) purchased from point electronic GmbH, Halle (Saale), Germany. Images were analyzed with the Digital Image Processing System (Version 2.6.13.0) purchased from point electronic GmbH, Halle (Saale), Germany.

Cell adherence on nanofiber nonwovens

L929 cells were seeded onto cover slips which had been coated with nanofibers in 12-well plates at a density of $5x10^4$ cells/well. Coated and uncoated glass cover slips were disinfected with 70 % isopropanol and dried under a laminar flow inside a clean bench. The dried glass cover slips were put in 12 well plates and L929 cells were seeded on the glass cover slips. After 48 hours the glass cover slips were removed from the medium and rinsed with PBS to make sure that only adherent cells remained on them. The cover slips were embedded using FluorSave Reagent (Calbiochem, San Diego, CA). Transmission light images were taken using a Zeiss Axiovert 100 M microscope (Zeiss, Oberkochen, Germany). Cell adhesion was determined by cell counting.

In a second experiment L929 cells were seeded onto cover slips which were coated with nanofibers in 12-well plates at a density of 10^5 cells/well. After 4 days the cells were lysed using cell culture lysis reagent (CCLR). The absolute protein content was determined by a BCA assay. 50 µl of the samples were mixed with 170 µl BCA reagent (BCA assay kit, Pierce, Rockford IL, USA) in 96 well plates. After 3 hours at 37°C and constant shaking the absorption at 560 nm was measured using a plate reader. All measurements were carried out in triplicate.

Cell viability on nanofiber nonwovens

Cell viability on nanofiber nonwovens was determined in triplicate by MTT assay according to [12]. L929 cells were seeded onto cover slips which were coated previously with nanofibers in 12-well plates at a density of 10^5 cells/well. After 4 days cell culture medium was aspirated and replaced by DMEM without serum containing 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Deisenhofen, Germany). After 4 h incubation at 37 °C in the dark, the medium was aspirated and formazan crystals were dissolved in 200 µl dimethylsulfoxide per well. Measurement was performed using an ELISA reader (Titertek Plus MS 212, ICN, Eschwege, Germany) at a wavelength of 570 nm and 690 nm.

Cell morphology on nanofiber nonwovens

L929 cells were seeded onto cover slips which were coated previously with nanofibers in 12-well plates at a density of $5x10^4$ cells/well. After 48 hours the cells were washed with PBS, fixed using 4% paraformaldehyde in PBS, cell nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) and the cells were washed again. Subsequently, cell membranes were labeled with fluorescein isothiocyanate-labelled wheat germ agglutinin (FITC-WGA). Finally

cells were embedded using FluorSave Reagent (Calbiochem, San Diego, CA). A Zeiss Axiovert 100 M microscope coupled to a Zeiss LSM 510 scanning device (Zeiss, Oberkochen, Germany) was used for confocal microscopy. The microscope was equipped with a Zeiss Neofluor 63*/1.2 objective. Excitation wavelengths were 364 nm (band pass filter 385 – 470 nm) for DAPI and 488 nm (long pass filter 505 nm) for FITC. Transmission images were obtained in the same scan.

Statistical analysis

All quantitative results were carried out at least in triplicate. Data is expressed as mean ± SD. Statistical analysis was carried out using an unpaired student's t-test. A value of $p < 0.05$ was considered to be statistically significant.

Results and discussion

In this study the technique of electrospinning of emulsions was applied to generate protein loaded NNs. All emulsions were composed of an organic PLLA solution in methylene chloride and an aqueous cytochrome C solution. The aqueous phase of the emulsions contained cytochrome C as a model substance for the incorporation of growth or differentiation factors which play an important role in tissue engineering. The successful incorporation of the protein and its controlled release from the NNs was already demonstrated in a previous publication [13]. The objective of this study was to clarify wether nanofiber nonwovens prepared via electrospinning of emulsions met the requirements for a tissue engineering scaffold. Suitable scaffolds for tissue engineering have to be biocompatible and biodegradable. They should physically resemble the natural structures of the extracellular matrix (ECM), promote cell adhesion, cell proliferation and support three dimensional cell growth.

The NNs of this study were composed of Poly(L-lactide) (PLLA), which is a biodegradable aliphatic polyester that is very popular for use in medical applications. PLLA L210 was chosen for the preparation of nanofiber nonwovens (NNs) since its rather high molecular weight facilitates electrospinning and the resulting fibers showed almost no swelling in aqueous environments. Furthermore, L-lactic acid, the product of PLLA hydrolysis, is biocompatible since it occurs naturally in the metabolic pathway of human beings. For these reasons, PLLA nanofibers have attracted the interest of numerous researchers in the field of tissue engineering and drug delivery [13-16].

Native page gel electrophoresis was used to check whether the electrospinning process would have a negative effect on the protein. There were no visible changes in retention time between native cytochrome C and the cytochrome C released from the NNs (data not shown). These results proved that neither degradation nor aggregation of the protein occurred during electrospinning. Other groups have also shown that electrospun proteins were stable and retained their bioactivity [17, 18].

Cell adhesion to artificial materials is affected by their surface properties such as wettability, roughness, surface charge and chemical functionalities [19]. Protein adsorption to polymeric surfaces is also an important factor since it usually takes place prior to cell adherence [20-22]. With respect to surface wettability of polymeric materials, cells effectively adhere onto surfaces presenting moderate wettability with water contact angles of 40 – 70° [23-27]. Static contact angles of water on PLLA NNs of this study were 109.4° ± 5.9, which showed their super hydrophobic surface characteristics. This phenomenon is also know for other nanofibrous structures [28]. To improve cell adherence the hydrophobicity of the PLLA NNs was reduced by addition of hydrophilic polymers like poly(ethylene imine) or poly(L-lysine) to the aqueous phase of the electrospinning emulsion. It was not possible to determine comparable contact angles of water on the NNs composed of these polymer blends, since the

NNs absorbed the water droplet very fast and easily. Charged hydrophilic polymers were chosen for blending, since they also increased the conductivity of the electrospinning emulsion. A higher conductivity facilitates electrospinning of these emulsions. The morphology of the resulting polymer blend fibers was similar to pure PLLA fibers. After analysis of the SEM images using the measuring tool of the "Digital Image Processing System" software it could be demonstrated that the mean fiber diameter increased significantly after addition of hydrophilic polymers with regard to the amount of added polymer.

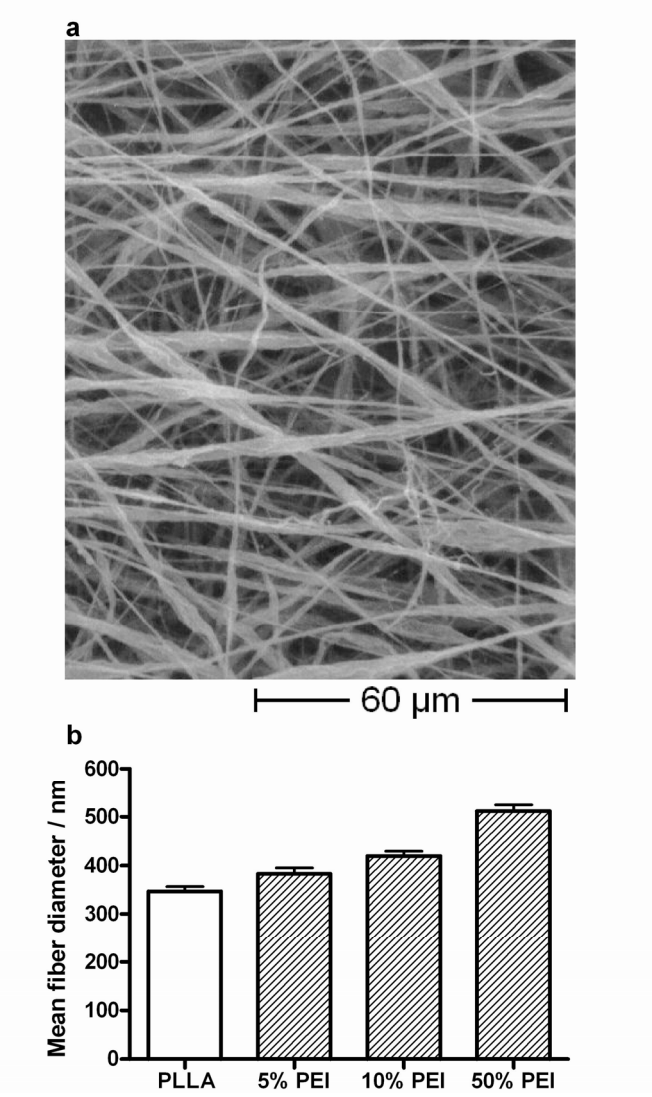

Figure 20: SEM image of PLLA nanofiber nonwoven (a) and analysis of mean fiber diameters of differently composed nanofibers (b).

Fibers electrospun from emulsions containing 2 wt% PLLA showed a mean fiber diameter of 346.6 ± 10.1 nm (Figure 1). The addition of 5% PEI to the aqueous phase of the emulsion led to a significant increase in mean fiber diameter to 383.4 ± 12.0 nm ($p < 0.05$). Further increases in PEI content in the blend fibers led to further increases in fiber diameter, with mean fiber diameters of 419.1 ± 10.8 nm at 10% PEI and 511.8 ± 13.1 nm at 50% PEI. It was expected that the addition of polyelectrolytes would increase charge density and decrease the fiber diameter [29]. In this case this effect was overcome by the increasing polymer concentration in the emulsion, which led to an increase in mean fiber diameter. The structural ECM proteins in native tissues have diameters of 50 – 500 nm. Since the diameter of our fibers ranged between 350 and 500 nm, the NNs produced by this electrospinning method perfectly matched the size of these structural proteins and should be very good in mimicking the native ECM.

The above mentioned results demonstrated that the NNs met the morphological requirements for a tissue engineering scaffold. In summary, our NNs were able to physically resemble the nanofibrous features of the ECM, they were biocompatible and biodegradable and we were able to incorporate bioactive substances. In a next step, interactions of a model cell line (L929) with these NNs were investigated to examine their biological suitability as tissue engineering scaffolds.

The first biological prerequisite for a suitable tissue engineering scaffold is to assure sufficient cell adhesion of the seeded cells. Only adhering cells are able to proliferate and differentiate to a tissue. For that reason, cell adhesion on different NNs was compared to the cell adhesion on a glass surface. Round glass cover slips were covered with nanofibers via electrospinning of emulsions. L929 mouse fibroblasts were seeded on theses cover slips and after 2 days cell adhesion was determined by cell counting on transmitted light images (Figure 2).

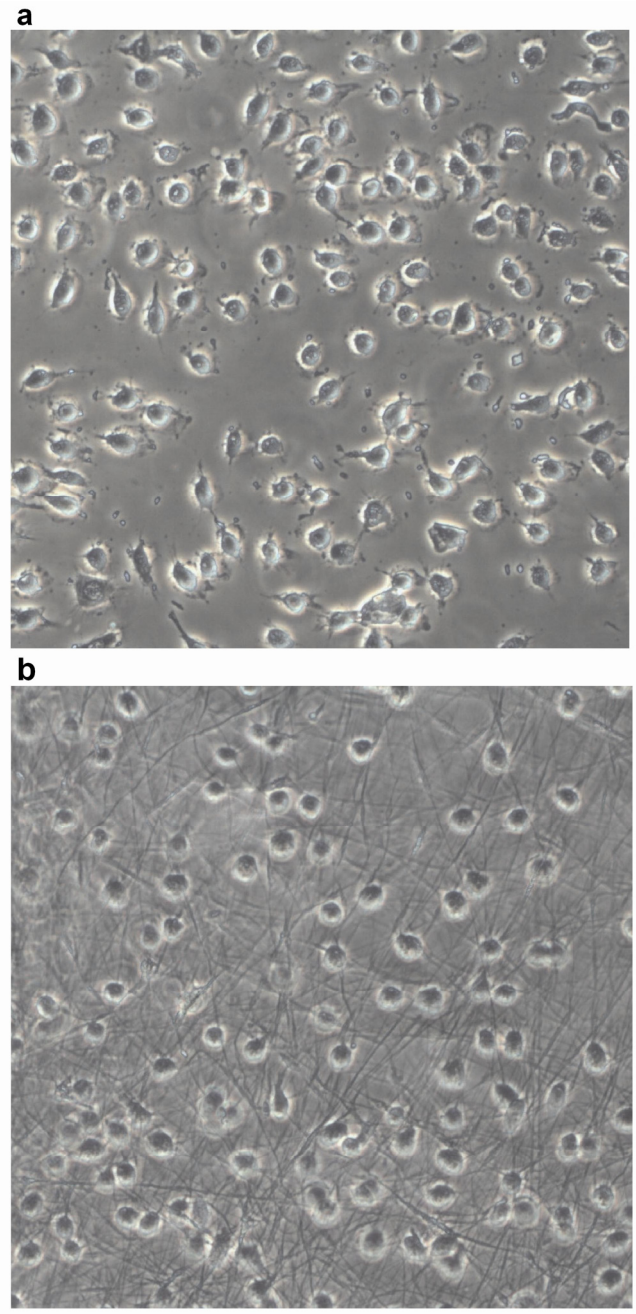

Figure 21: L929 cells grown on a glass surface (a) and cells grown on a PLLA nanofiber nonwoven.

Using these procedures we compared cell adherence on a glass surface with nanofibers consisting of either pure PLLA or blends of PLLA and hydrophilic polymers. As mentioned previously, the addition of PEI or PLL should primarily decrease the hydrophobicity of the NN surface. For that reason, the blends were composed of only 5 or 10% of the hydrophilic polymers. Furthermore the addition of positive charges to the NNs should lead to higher cell

adherence through electrostatic attraction. It is commonly know, that cell membranes are negatively charged and a positively charged surface should therefore be more attractive for cell adherence than a neutral or negatively charged surface.

Our results showed that cell adhesion on all fibers was as good as on a glass surface. Moreover, the number of cells adhering on nanofibers consisting of 95% PLLA and 5% PLL was significantly higher than on a glass surface or pure PLLA nanofibers (Figure 3a). Allowing the cells to grow for more than two days, led to a cell density which made it impossible to count their number on transmitted light images. A common experiment to determine cell density would be the detachment of the seeded cells from the surface and counting of the cell number using a hemocytometer. Usually the treatment of adhering cells with trypsin leads to the detachment of the cells. But cells grown on NNs were so strongly attached to the NNs that even high concentrations of trypsin and long incubation times did not lead to a detachment of the cells. This might by due to tight cell-matrix connections, which also occur in natural tissues between cells and the ECM. These adhesion sites are termed focal contacts or focal adhesions and are integrin-based structures that mediate strong cell-substrate adhesion [30]. These filament-like structures of 10-15 nm length extend from the cell body and attach to the nanofibers [14, 31].

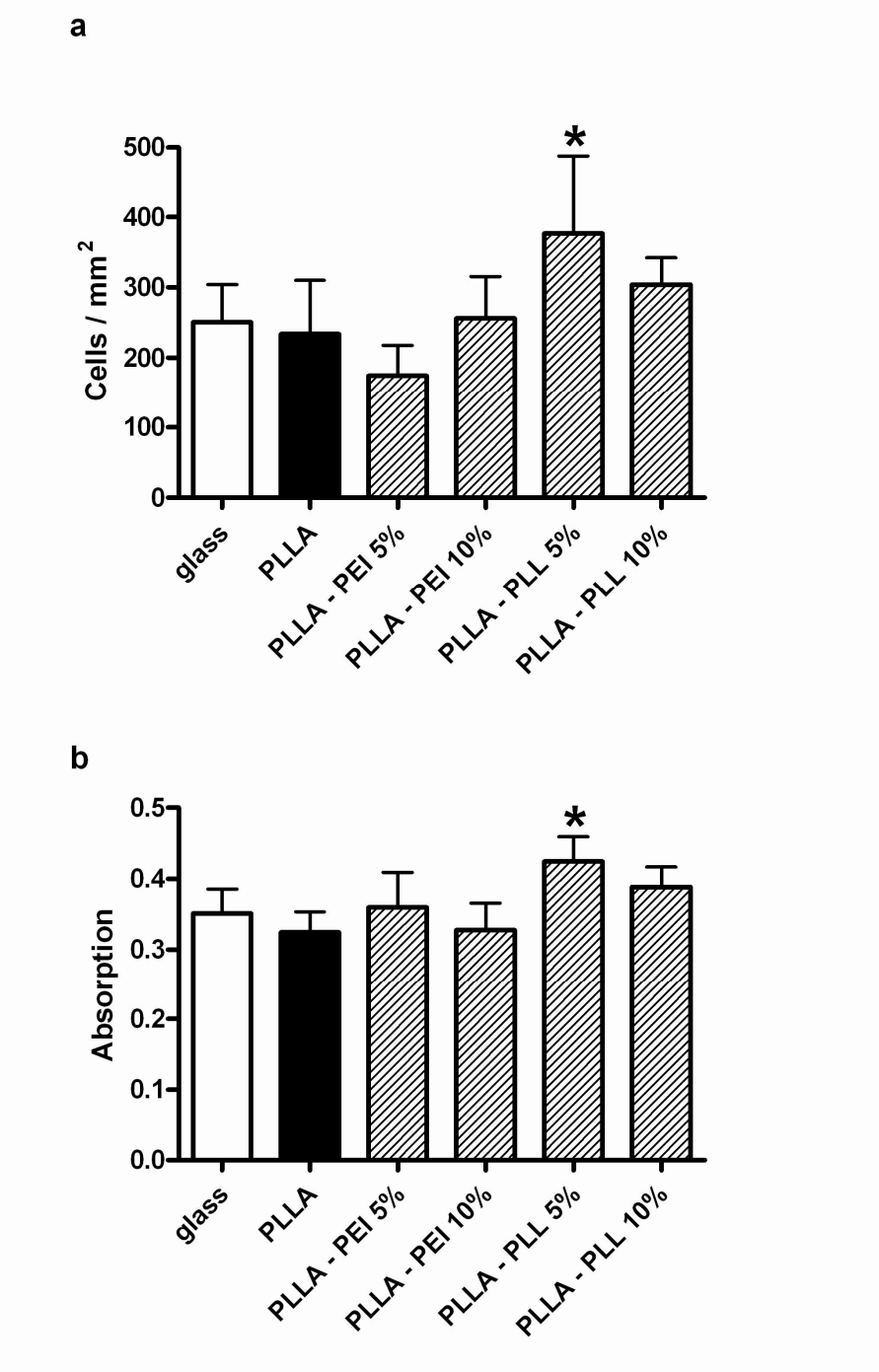

Figure 22: Comparison of cell adherence on different surfaces determined by cell counting on transmitted light images (a) and calculated via protein quantification (b).

Another experiment was carried out, which should on the one hand verify the results of the cell counting experiment via a second detection method and on the other hand should monitor cell adherence after a longer period of time. Round glass cover slips were coated with the same composition of nanofibers like in the first experiment. After 4 days the glass cover slips

were removed from the medium and rinsed with PBS to make sure that only adherent cells remained on them. The glass cover slips were put in 12 well plates and the adherent cells were lysed using cell culture lysis reagent (CCLR). After that, the protein content per well was determined via a BCA assay. It is obvious that a higher cell density led to higher protein content, which provided the possibility of an indirect determination of the cell adherence after 4 days. The outcome of this experiment confirmed the findings of the cell counting experiment. There were no significant differences in cell adherence between a glass surface and NNs except for nanofibers consisting of 95% PLLA and 5% PLL, which showed a significantly higher cell adherence compared to glass or pure PLLA nanofibers (Figure 3b).

Since neither the transmitted light images nor the protein content gave evidence whether the cells are still alive, we determined cell viability on coated and uncoated cover slips via a MTT assay. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. Cell viability on the glass surface was normalized to 1.0 and cell viabilities on the different NNs were related to the cell viability on the glass surface. The results in figure 4 demonstrate that there were no significant differences in cell viability between a glass surface and the NNs.

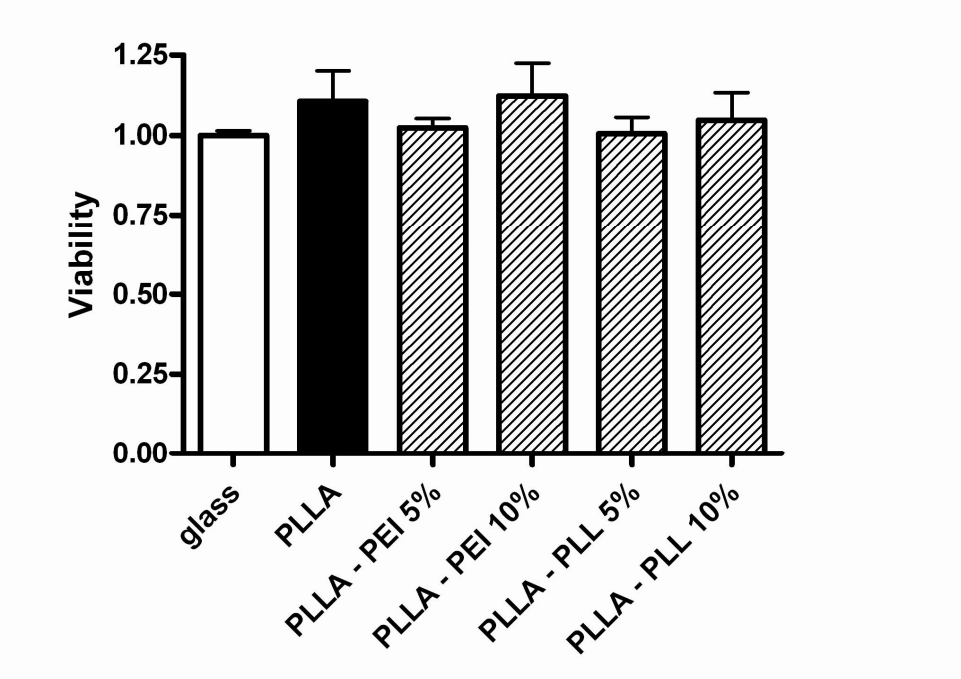

Figure 23: Cell viability on different surfaces determined via MTT assay.

After cells started to adhere on a surface, they will usually begin to spread on this surface. This is an essential step towards cell differentiation and proliferation. Confocal laser scanning microscopy was used to take a closer look on the morphology of the adhering cells on different surfaces. Cell nuclei were labeled with 4,6-diamidino-2-phenylindole (DAPI), which made them appear blue on the images. Cell membranes were stained with fluorescein isothiocyanate-labelled wheat germ agglutinin (FITC-WGA), which made them appear green. In this experiment cell morphologies of adhering cells on 4 different surfaces were compared. These surfaces were glass, a PLLA film on glass, PLLA nanofibers, and PLLA-PLL 5% nanofibers. Figure 5a shows the morphology of L929 cells which were seeded on a glass surface after 48 hours. All cells spread on the glass surface in two or more directions. Figure 5b shows cell morphology on a PLLA film. Many cells on the PLLA film showed a spherical morphology and only some a spindle like structure. The PLLA film was rather hydrophobic and this attribute limited spreading of the cells. The small spherical structures on this picture were air inclusions which were generated during manufacturing of the film via spincoating.

As mentioned before, pure PLLA nanofiber nonwovens exhibited a super hydrophobic surface. This is the reason why the L929 cells on PLLA nanofibers were almost completely spherical and showed only small signs of spreading (Figure 5c). The addition of PLL decreased the hydrophobic features of the fibers and led to a spreading behavior which was as good as on a glass surface. In contrast to PLLA film or fibers, all cells spread on the surface of PLLA-PLL 5% fibers in two or more directions.

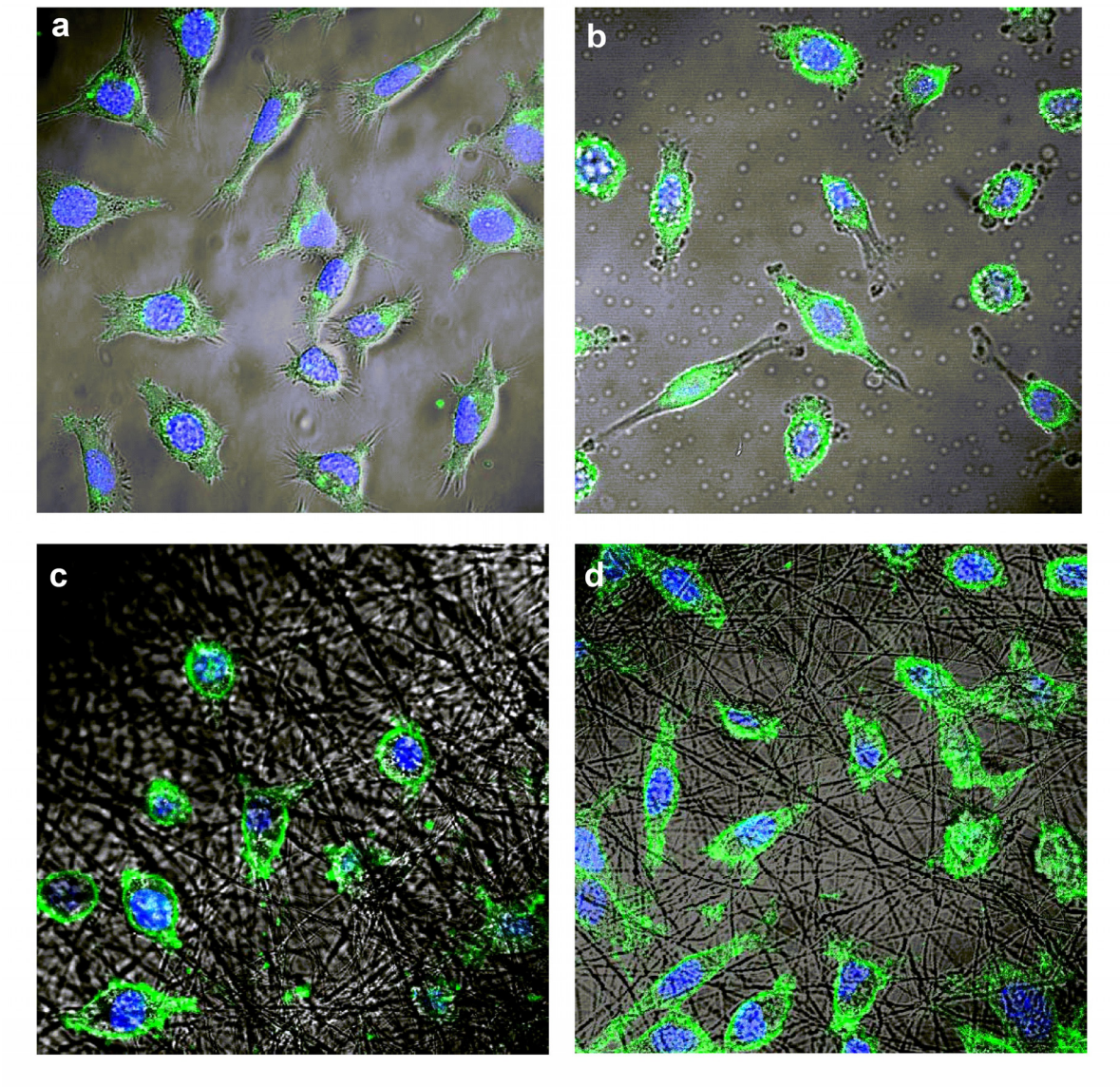

Figure 24: CLSM images showing morphology of cells grown on different surfaces (a: glass, b: PLLA film, c: PLLA nanofiber nonwoven, c: PLLA – PLL 5% nanofiber nonwoven).

One major prerequisite for a tissue engineering scaffold is the support of three dimensional growth for the seeded cells. Confocal laser scanning microscopy is able to produce images of various z axis planes of the sample. By putting several images of different planes from the same sample on top of each other, a 3 dimensional view of the sample was assembled. Using this procedure, cross-sections of fluorescence labelled cells on nanofiber nonwovens were obtained.

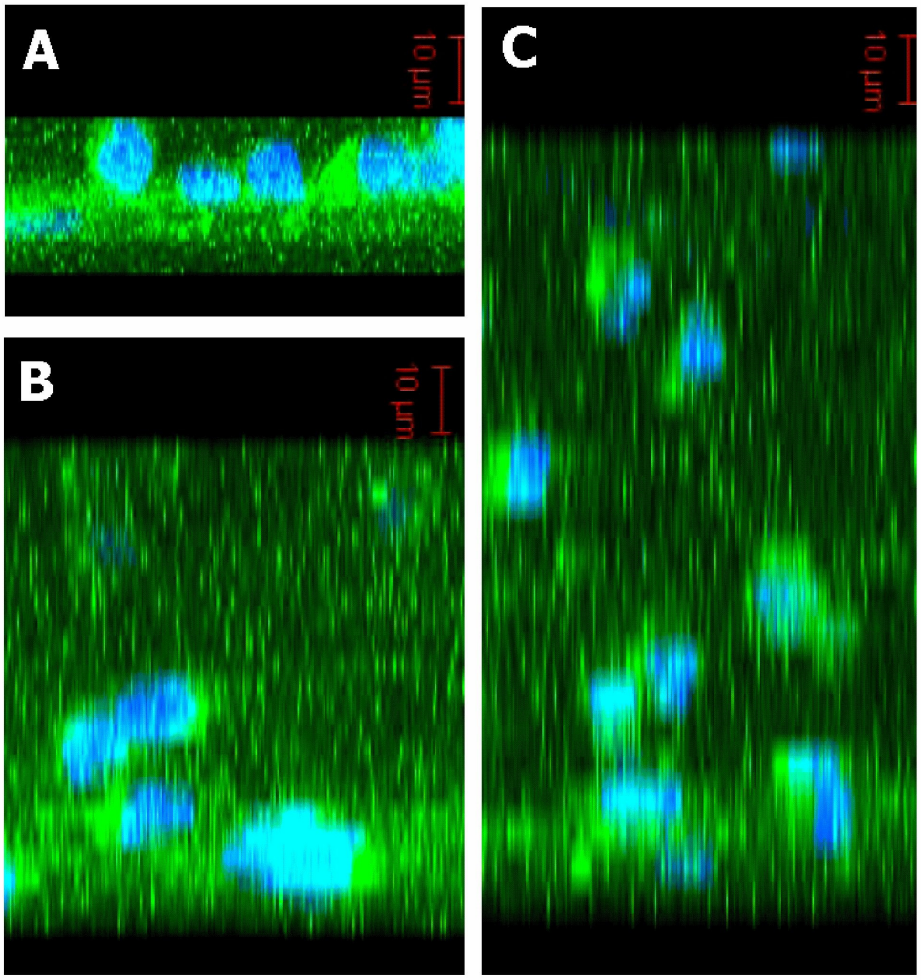

Figure 25: Z-stacks of CLSM images showing cross-sections of L929 cell nuclei (blue) grown on different nanofiber nonwovens (A: PLLA NNs, C: PLLA – PLL 5% NNs, C: PLLA – PLL 10% NNs)

Figure 6 shows the cross-sections of PLLA NNs (A), PLLA-PLL 5% NNs (B), and PLLA-PLL 10% NNs (C). The glass cover slip, which held the NNs is located at the bottom of each image. The blue spherical structures represent the cell nuclei of L929 cells. FITC-WGA was

originally used for staining of cell membranes, but it also adhered to the fibers to a small extend. In this way it was possible to estimate the thickness of the different NNs. The images proved that the addition of hydrophilic polymers led to water uptake and swelling of the NNs. This swelling behaviour depended on the amount of added hydrophilic polymer. A higher fraction of PLL led to a higher water uptake and swelling. Nevertheless the cells were still adhering to the nanofibers and were growing throughout the whole fibrous network. This gave the cells the opportunity to proliferate in all 3 dimensions and demonstrated the ability of the NNs to support three dimensional growth.

Conclusion

In this study, we succeeded in manufacturing protein loaded and biodegradable nanofiber nonwovens for the application in tissue engineering. It was demonstrated that the nanofibrous structure of this scaffold was similar to the natural ECM regarding fiber diameter. Favorable interactions between L929 cells and the scaffold were demonstrated by cell adhesion, cell viability and cell morphology studies. Furthermore, the addition of hydrophilic polymers gave us the opportunity to tailor the properties of these nanofiber nonwovens. Nanofiber nonwovens composed of 95% PLLA and 5% PLL featured the most promising characteristics. These results strongly suggest a huge potential of these nanofiber nonwovens for their application in tissue engineering.

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Chapter 5:

A short overview on microencapsulation techniques based on phase separation and coacervation

Published in *Drugs and the Pharmaceutical Sciences* Vol. 158
„Microencapsulation 2nd edition“ (2006) pp: 99-106

Microencapsulation has been widely used in many industrial applications, including graphics, food and agriculture. Application of microparticulate delivery systems for pharmaceutical and medical problems has also been extensively studied. Microencapsulation technology allows protection of the drug from the environment, stabilization of sensitive drug substances, elimination of incompatibilities or masking of unpleasant taste. Microparticles are particularly interesting for the development of controlled or prolonged release dosage forms. They play an important role as drug delivery systems aiming at improved bioavailability of conventional drugs and minimizing side effects.

Pharmaceutical applications of microspheres comprise different routes of administration among them oral, pulmonary, and parenteral delivery can be distinguished. Microparticles are consequently administered either as dry powders, e.g. by inhalation or in form of an aqueous suspension, e.g. by injection. Our considerations will be mainly limited to the parenteral route of administration.

The term “microencapsulation” is used to designate a family of technologies used to entrap solids, liquids or gases inside a polymeric matrix or shell. In contrast to film coating techniques, particle formation occurs in one single step. Usually the drug substance is encapsulated in a biocompatible or biodegradable polymer forming particles with a diameter in the size range of 1 - 1000 μm. As parenteral delivery system the diameter of the microparticles should be <250 μm, ideally <125 μm, to allow injections with acceptable needle diameters.

Two general micro-morphologies of microparticles can be distinguished: microcapsules and microspheres. The term “microcapsule” should be used to describe particles in which a drug containing core is completely surrounded by a polymer shell. The core can be solid, liquid or gas; the shell is a continuous, porous or non-porous, polymeric layer. On the other hand “microspheres” are defined as microparticles in which the drug substance is either

homogeneously dissolved or dispersed in a polymeric matrix. These monolithic delivery systems can also be described as solid solutions or solid dispersions of the drug in a micro-scale polymeric matrix. Microspheres show different release properties compared to true microcapsules and an additional feature is that catastrophic drug burst due to rupture of the shell cannot occur.

With the recent progress in biotechnology and genetic engineering a large number of protein drugs have found therapeutic application. In most cases these bioactive agents are ineffective after peroral administration since they are rapidly degraded and deactivated by proteolytic enzymes in the gastro-intestinal tract. Furthermore their molecular weights are usually too high for oral absorption. Due to their short biological half-lives, frequent injections or infusion therapy are needed to achieve therapeutic plasma levels for prolonged periods of time. Biodegradable parenteral delivery systems, such as implants or microparticles have proven their suitability as parenteral depot systems for the administration of proteins and polypeptides. DNA and oligonucleotides are another challenging class of therapeutic agents which have also been successfully encapsulated.

In this chapter we describe microencapsulation techniques which are relevant for the preparation of microparticles for parenteral depot delivery systems on an industrial scale. After a short summary of the most common biodegradable polymers which are used for this purpose, different techniques will be explained in more detail and examples will be given of pharmaceutical dosage forms prepared by these methods.

Biodegradable Polymers

Many different polymers are used in pharmaceutical sciences and technology as excipients, drug delivery systems, bandage-, suture- or packing materials. For parenteral depot systems, at first synthetic non-degradable polymers were used as drug carriers, diffusion barriers or

protective coatings. But since the 1970s new polymer materials emerged, degrading in a biological environment. For drug delivery a biodegradable polymer and its degradation products have to be biocompatible and toxicologically harmless. Biodegradable polymers are advantageous for all those cases, where the removal of the device is inconvenient or even impossible.

Biodegradable polymers were first used as biomaterials for the manufacturing of absorbable sutures [1-3] and orthopaedic fixture materials [4]. After that they attracted a great deal of attention for drug delivery [5] and tissue engineering [6]. Since then the search for new biodegradable polymers progressed to design polymers for specific applications [7].

Biodegradable polymers can be classified based upon the mechanism of erosion. It is essential to recognize that “degradation” refers to the chemical process of bond cleavage whereas “erosion” is a physical phenomenon dependent on dissolution and diffusion processes. Two mechanisms of polymer erosion can be distinguished: surface and bulk erosion. Surface eroding devices degrade by enzymatic or hydrolytic cleavage starting from the surface. In an ideal case the interior of the device contains unchanged polymer in the dry state. Bulk eroding devices degrade from the “inside” and hence water uptake precedes degradation of the polymer mostly by hydrolytic cleavage reactions. As a consequence the molecular weight of the polymer decreases until the cleavage products become water soluble. The interior of the devices retains drug and cleavage products in an aqueous milieu. In many cases both mechanisms occur concurrently, but the relative extend of surface or bulk erosion varies depending on the structure and composition of the biodegradable polymer. Surface erosion is considered most desirable, because the kinetics of erosion, and hence the rate of drug release, is more predictable and the drug substance is not exposed to an acidic environment generated by hydrolytic cleavage products from e.g. polyesters. The erosion mechanism depends in particular on the rate of water permeation in comparison to the rate of bond cleavage and

consequently diffusion of degradation products. In case water permeates faster into the polymer matrix than erosion takes place, bulk erosion can be expected. On the other hand surface erosion occurs if the rate of erosion exceeds the rate of water permeation into the bulk of the polymer.

Controlled release dosage forms improve the effectiveness of drug therapy by increasing therapeutic activity compared to the intensity of side effects or reducing the number of drug administrations required during treatment. A variety of biodegradable polymers have been synthesized for the controlled release of different drugs. The selection and design of a suitable biodegradable polymer is the first challenging step for the development of a parenteral drug delivery system. Several classes of synthetic polymers have been proposed, including poly(ester)s, poly(anhydride)s, poly(carbonate)s, poly(amino acid)s, poly(amide)s, poly(urethane)s, poly(ortho-ester)s, poly(iminocarbonate)s and poly(phosphazene)s. In the following part the most common classes of biodegradable polymers are briefly introduced. A detailed discussion is beyond the scope of this chapter and the reader is referred to the literature.

Poly(ester)s

The most widely investigated class of polymers with regard to toxicological and clinical data comprise aliphatic poly(ester)s consisting of lactic and glycolic acid. Their corresponding polymers, poly(lactic acid), poly(glycolic acid) and copolymer poly(lactic-co-glycolic acid) have found wide-spread commercial application as drug delivery devices. Some of these drug delivery devices will be presented later in this chapter.

Since high molecular weight polymers of glycolic and lactic acid cannot be obtained by condensation of the related hydroxycarboxylic acids, the polymers are usually produced by ring-opening polymerization of their cyclic dimers (Figure 1).

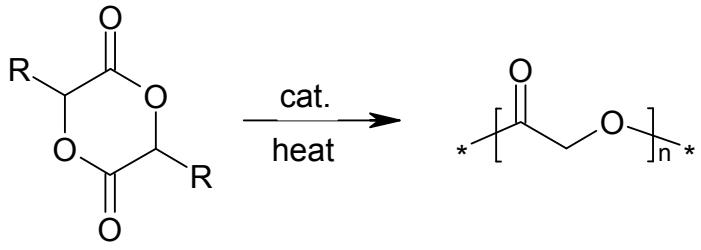

Figure 1: Ring-opening polymerization of poly(lactide) (R=H) or poly(glycolide) (R=CH_3)

Lactic acid contains an asymmetric α-carbon which is typically described as D or L form and thus three different polymers are conceivable: poly(L-lactic acid), poly(D-lactic acid) and poly(DL-lactic acid). To date, poly(L-lactide) and poly(DL-lactide) have received the most attention among these poly(lactide)s. The stereochemistry of the polymers affects their mechanical, thermal and biological properties. Poly(lactide-co-glycolide) represents the "gold standard" of biodegradable polymers. The properties of these copolymers can be modified by changing the ratio of poly(lactide) and poly(glycolide).

The mechanism of degradation in poly(ester)s is classified as bulk degradation with random hydrolytic scission of the ester bond linkages in the polymer backbone. In larger devices the acidic degradation products can accumulate in the polymer matrix due to reduced diffusion. This leads to an autocatalytic effect on the degradation of the device.

Another polymer with excellent biocompatibility is poly(ethylene glycol). One reason for its high biocompatibility is the hydrophilic nature of poly(ethylene glycol). This attribute leads to formation of hydrogen bonds between water and the polymer chains which also inhibit protein adsorption. Thus poly(ethylene glycol) chains at the surface of a parenteral device lead to increased blood circulation times by prolonging biological events such as endocytosis or phagocytosis. Furthermore, block copolymers of poly(ethylene glycol) and poly(lactide) or poly(lactide-co-glycolide) have been synthesized for the encapsulation of proteins [8].

Macromolecules which are incorporated into devices made from these copolymers are less likely to adsorb to the delivery system through hydrophobic interactions.

Poly(anhydride)s

Poly(anhydride)s are more hydrophobic than poly(ester)s and yet contain water sensitive bonds. This reduces the water permeation into the polymer bulk and hence poly(anhydride)s are believed to undergo predominately surface erosion by cleavage of the anhydride bonds at the surface of the device. The most widely studied poly(anhydride)s are based on sebacic acid, p-(carboxyphenoxy)propane and p-(carboxyphenoxy)hexane (Figure 2). Sipos et al. were able to improve the interstitial administration of an antitumor agent using a loaded polymer disc composed of poly(carboxyphenoxypropane-sebacic acid) [9].

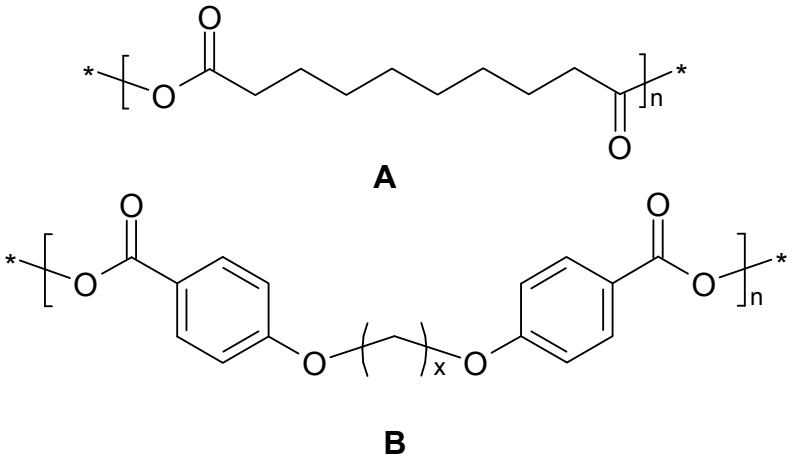

Figure 2: Structures of Poly(anhydride)s based on monomers of sebacic acid (A), p-(carboxyphenoxy)propane (B for x = 3) and p-(carboxyphenoxy)hexane (B for x = 6).

For the design of parenteral drug delivery systems the selection of the biodegradable polymer is a very important consideration because solubility properties define the scope of microencapsulation technologies that can be used on one hand and the drug release and degradation properties on the other hand. It should be noted that the biodegradable polymer is an integral part of the device and should not be regarded as an inert excipient. Different

microencapsulation techniques were used to manufacture parenteral delivery systems on a commercial scale. These techniques can be classified as WOW-double emulsion method, phase separation techniques and spray drying. Phase separation and coacervation will be discussed in detail in the following section.

Phase separation and coacervation

Carbonless copy paper was the first commercially successful product containing microcapsules. The original microencapsulation technique to produce these pressure-sensitive dye microcapsules was described by Green and Schleicher in the 1950s and is based on macromolecular coacervation [10, 11]. The Dutch scientists Bungenberg de Jong and Kruyt introduced the term "coacervation" in 1929 [12]. It is derived from the Latin word "coacervus" which means heap or pile. Coacervation describes macromolecular aggregation processes brought about by partial desolvation of fully solvated macromolecules. A distinction is drawn between "simple coacervation" and "complex coacervation". In simple coacervation processes phase separation is induced by addition of alcohol or salt, temperature change or pH change. In complex coacervation an oppositely charged polymer is added to the polymer solution leading to the formation of a coacervate phase via anion-cation interactions.

These phase separation processes can be used to encapsulate solid or liquid drug particles which are dispersed in the polymer solution. The encapsulation process involves three main stages (Figure 3):

1. Phase separation of the primary wall polymer solution is induced by one of the above mentioned methods. This leads to a three phase system consisting of a polymer-rich liquid phase (coacervate phase), a polymer-lean liquid phase and a solid or liquid

phase made up of the drug particles. This suspension or emulsion is usually maintained by continuous agitation.

2. The polymer-rich phase deposits as microdroplets on the interface of the dispersed drug particles. After that the microdroplets start to spread which leads to fusion into a membrane.
3. The originated polymer membrane is hardened through thermal, desolvation or chemical methods.

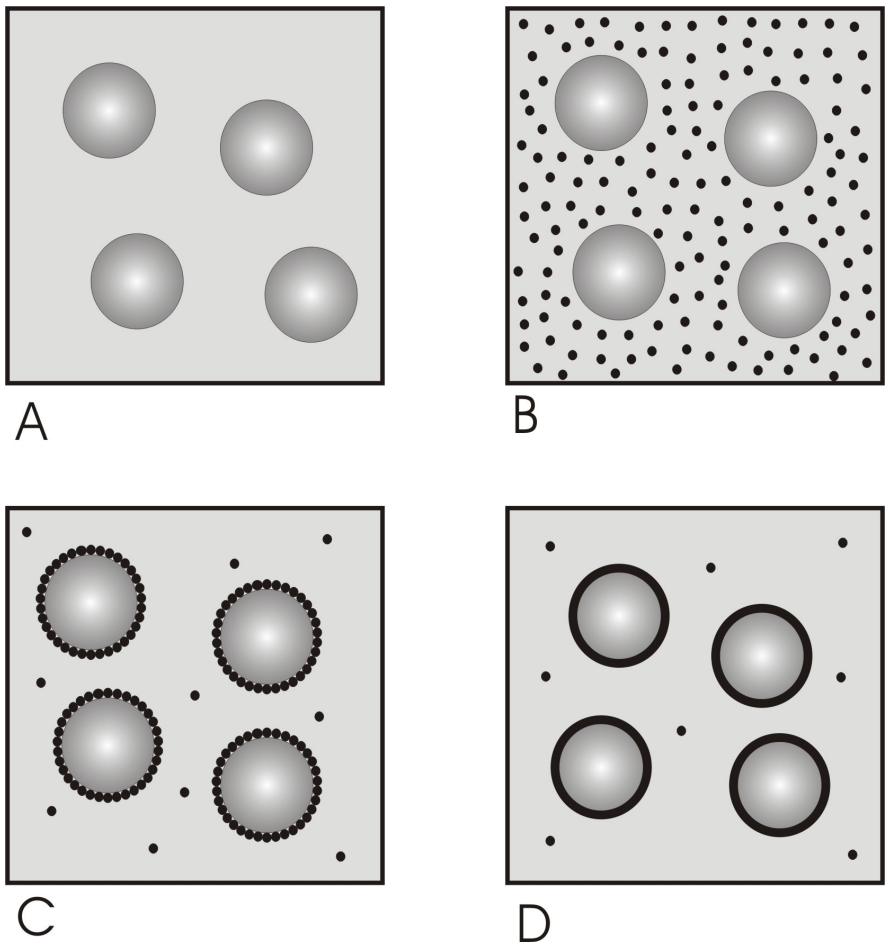

Figure 3: Schematics representation of microencapsulation through coacervation: (A) dispersed liquid or solid drug particles; (B) induction of phase separation; (C) deposition of microdroplets at the surface; (D) fusion into a membrane.

A simple coacervation process can easily be demonstrated in an aqueous gelatin solution in which the solid or liquid drug is dispersed. Dropwise addition of a competing substance of greater hydrophilicity or concentration, such as an alcohol, will lead to phase separation resulting in the two liquid phases: one phase in which the concentration of gelatin is high and a second phase with a low gelatin concentration. This phase separation process takes place because the water-alcohol interactions in the gelatin solution are more favorable than the water-gelatin interactions. As a result the macromolecules are partially desolvated. This, in turn, leads to increased interactions between gelatin molecules themselves, and hence to the formation of macromolecular aggregates or coacervates. The individual polymer coacervates form the polymer-rich coacervate phase which deposits on the interface of the dispersed solid or liquid drug particles.

The coacervation of gelatin and gum arabic (acacia) is an example of a complex coacervation process. The first step is to prepare a solution of gelatin and a solution of gum arabic in 45-55°C water. The drug is dispersed or emulsified in the gelatin solution at above 45°C. The resulting suspension or emulsion is then diluted by addition of water and gum arabic solution. After that the pH is adjusted to below the isoelectric point of gelatin (3.8 - 4.4) so that its charge becomes positive while that of the acacia is negative. At this point the system is allowed to cool down to room temperature while being stirred. During this cooling process, the liquid coacervate slowly deposits around the drug particles forming microparticles. As a last step the mixture is cooled below 10 °C and glutaraldehyde is added to crosslink the polymers and to harden the polymer shell.

Gelatin and acacia are both water soluble and hence the above mentioned classical methods are referred to as aqueous coacervation techniques. Since the use of water-insoluble polymers (e.g. many biodegradable polymers) in pharmaceutical technologies increased, new non-aqueous coacervation techniques have been developed. These non-aqueous coacervation

techniques are also referred to as organic phase separation. Desolvation is induced by temperature change, addition of a low molecular weight non-solvent for the polymer or in most cases by polymer-polymer incompatibility.

If two chemically different polymers are dissolved in a common solvent they usually separate into two phases as a consequence of incompatibilities. One phase is rich in one polymer, whereas the second phase is rich in the second polymer. For microencapsulation the polymer that serves as the capsule shell is dissolved in an organic solvent. Then the material to be encapsulated is introduced and a suspension or an emulsion is formed. To this mixture the second polymer solution is added to induce phase separation by polymer-polymer incompatibility. The desired coating polymer has to deposit preferentially on the surface of the drug for successful microencapsulation. After that the mixture is usually cooled down and the embryonic capsule walls are either chemically cross-linked or hardened by removal of the solvent.

Application in pharmacy

The preparation of microcapsules by coacervation for pharmaceutical purposes started in the 1960s. Phares and Sperandio were one of the first to investigate the phenomenon of coacervation as a new way of coating pharmaceuticals [13, 14]. They used a simple coacervation method to encapsulate five solids and two liquids by addition of sodium sulfate to an aqueous gelatin solution. Among those solids were drugs like acetylsalicylic acid and procaine penicillin G. Nixon, Khalil and Carless investigated the role of pH and the effect of electrolytes on coacervation in the systems gelatin-water-ethanol and gelatin-water-sodium sulfate. They then presented an improved aqueous simple coacervation method for microcapsules containing sulphamerazine [15-17]. Luzzi and Gerraughty used the classical aqueous complex coacervation technique with gelatin and acacia to produce microcapsules

containing pentobarbituric acid and investigated the effect of additives and formulation techniques on controlled release of the drug from the microcapsules [18, 19].

Other polymeric systems have also been described for the preparation of microcapsule products via aqueous coacervation techniques. Merkle and Speiser prepared phenacetine-loaded microcapsules by simple coacervation of a cellulose acetate phthalate solution through addition of sodium sulfate [20]. Mortada et. al. used gelatin and Gantrez-AN, a hydrolyzed copolymer of maleic anhydride and methyl vinyl ether, for a complex coacervation technique to produce nitrofurantoin-loaded micrcapsules [21, 22].

Today biodegradable polymers like poly(lactide) (PLA) and poly(lactide-co-glycolide) are the most attractive polymers for microencapsulation of bioactive drugs like peptides, proteins and hormones. Since these polymers are water-insoluble, many organic phase separation techniques have been developed. The first method to prepare polyester microspheres was described probably for the first time in 1978 [23]. In the early 1980s the luteinizing hormone-releasing hormone analogue nafarelin was encapsulated into poly(D,L-lactic-co-glycolic)acid by a phase separation process [24, 25]. These microspheres represented one of the first industrial developments of a controlled peptide release system for parental application. Since then, a great variety of peptide and protein compounds have been investigated.

Two commercially available pharmaceuticals containing microparticles which have been prepared by a phase separation technique are Decapeptyl® Depot (Ferring Pharmaceuticals) and Sandostatin®-LAR® Depot (Novartis Pharma). Decapeptyl® Depot is composed of triptorelin-loaded PLGA-microspheres for the treatment of prostate cancer of men [26] or the treatment of infertility of women. Sandostatin®-LAR® Depot consists of octreotide acetate-loaded PLGA-microspheres for the treatment of acromegaly, a disease resulting from a growth hormone secreting pituitary adenoma [27, 28].

Long-term treatment of acromegaly with octreotide would require three subcutaneous injections a day. Comets et al. described the absorption profiles of octreotide pamoate-loaded microspheres (Figure 5) in comparison to an i.v. application of octreotide solution (Figure 4) [29].

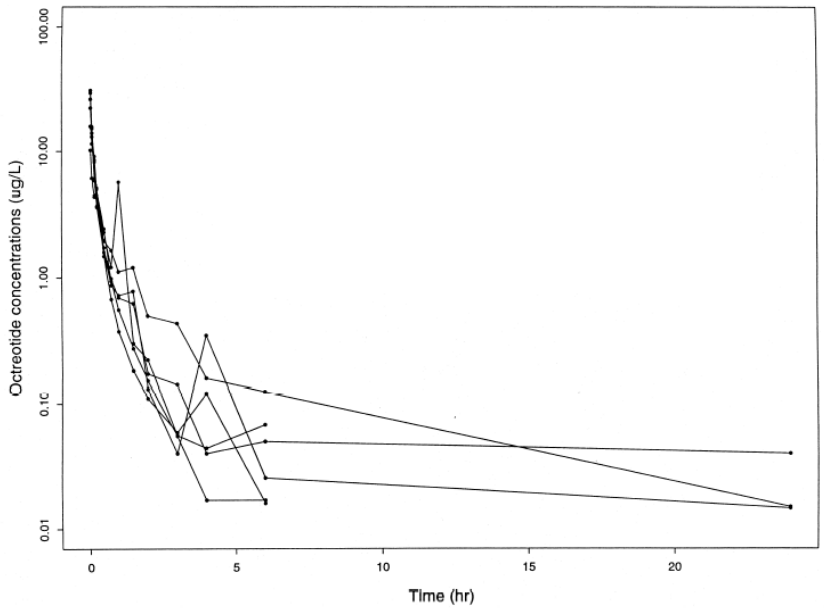

Figure 4: Plasma levels of octreotide after an i.v. bolus injection of 0.025 mg in six rabbits.

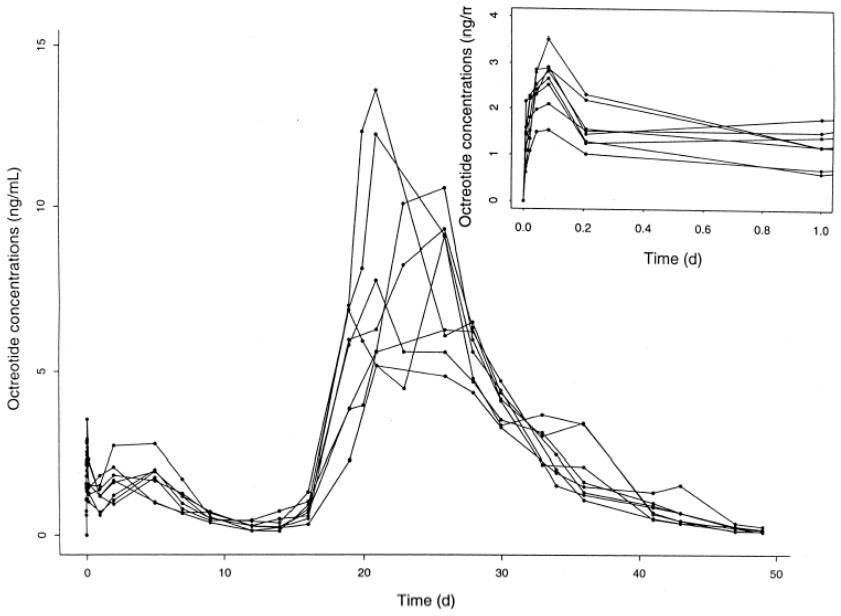

Figure 5: Plasma levels of octreotide after an i.m. bolus injection of 5 mg/kg of octreotide pamoate-loaded microspheres in eight rabbits. The inset shows the data collected during the first day of the experiment.

For i.v. bolus injection sufficient plasma levels of octreotide can only be achieved for a few hours. The release of octreotide from the microparticles leads to a triphasic absorption profile. During the initial phase about 5% of the drug is released. After a lag-phase the major part of the drug is released. A high interindividual variability especially in the magnitude of the absorption can be observed.

Octreotide release from microspheres was studied under in vitro conditions [30]. After 27 days more than 90% of the drug was released. The release profile depended on the ionic strength of the medium (Figure 6). In vivo a drug release for more than 40 days was observed (Figure 7). The data highlights the problem of "in vitro-in vivo-correlation". A direct prediction of the in vivo release based on in vitro data is not possible in most cases.

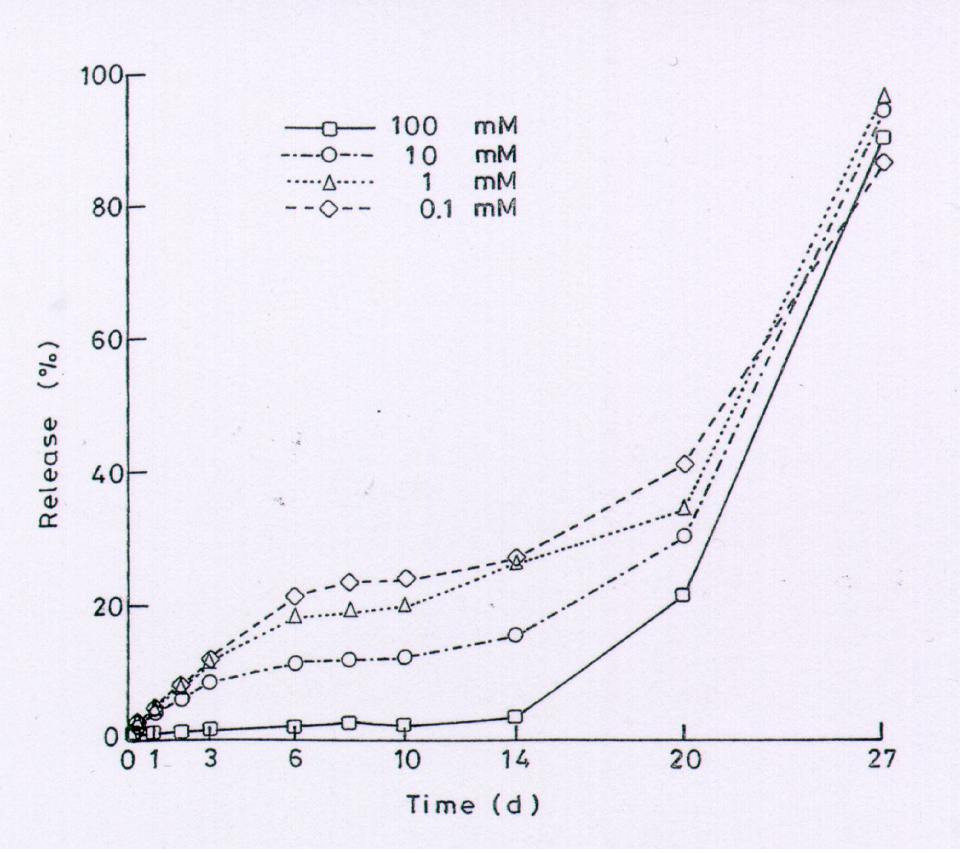

Figure 6: Cumulative in vitro release from octreotide loaded microspheres in acetate buffer with different salt concentrations.

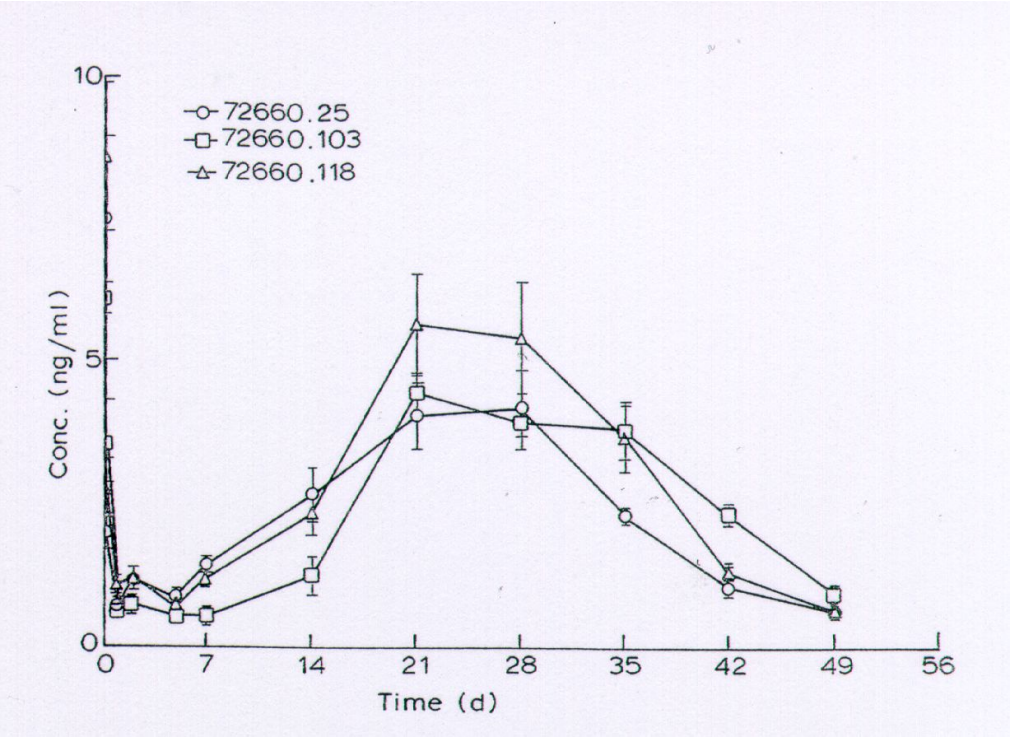

Figure 7: Octreotide plasma levels of rabbits after i.m. application of 5 mg/kg octreotide loaded microspheres (three different formulations)

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Chapter 6:

Development of new microencapsulation techniques based on biodegradable polymers and ionic liquids

Summary:

The objective of this study was to develop a new microencapsulation technique which uses an ionic liquid (IL) as one common solvent for a biodegradable polymer and a hydrophilic macromolecule. Biodegradable Polymers were sufficiently soluble in ILs, but hydrophilic macromolecules did not show sufficient solubility. Nevertheless the microencapsulation of bovine serum albumin (BSA) was possible via a phase separation technique, but encapsulation efficiencies were rather low. The size of the microparticles ranged between 25 - 30 µm. Additionally, several dyes were tested for their solubility in ILs and the possibilities to encapsulate them as model compounds. Mean particle diameters, encapsulation efficiencies and release profiles for the dyes were determined. Several variations were applied to the microencapsulation technique in order to increase encapsulation efficiency for the dyes. A MTT assay was carried out to determine the cytotoxicity exemplary for one IL. Although we were not able to find an IL among the tested ones, which is capable of dissolving a biodegradable polymer and a hydrophilic macromolecule, this goal might be easily achievable with another IL.

Introduction:

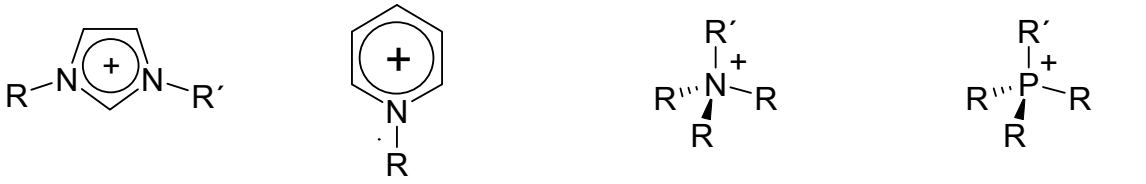
Ionic liquids (ILs) are defined as salts having melting points below 100°C. ILs which are liquid at room temperature are preferably used for most applications. Drawing the line between salts and ILs at 100°C is justified by their broadened application range below that temperature. In contrast to molten salts, which are usually highly viscous and corrosive, ILs have low viscosities and are stable. They make up a new class of ionic solvents having remarkable characteristics. An IL is composed of at least 2 different molecules, an anion and a cation. They are nonflammable, thermally stable and nonvolatile. These properties make ILs very interesting as replacements for volatile organic compounds to achieve resource saving and environmentally harmless processes [1]. Furthermore, the use of ILs as solvents can lead to remarkable improvements in synthesis and catalysis [2, 3], liquid chromatography [4, 5] or extraction processes [6-8].

Walden published the first IL in 1914, which was Ethylammoniumnitrate ($Et\text{-}NH_3^+$ NO_3^-) with a melting point at 12°C [9]. In 1948 Hurley und Wier were the first to use ILs as solvents, in this case for the electrodeposition of aluminium [10]. At the beginning ILs were primarily used in electrochemical processes [11]. These ILs were based on alkylpyridinium chloroaluminates A considerable drawback of these chlororaluminate based ILs was their need to protect them rigorously from moisture and other oxide impurities. In 1992 Wilkes et. al. introduced air and water stable imidazolim based ionic liquids, which are most commonly used until today [12]. Based on these results many new cation/anion combinations have been reported as ILs [13-15]. Even DNA is reported to have an IL form when combined with certain cations [16].

The synthesis of an IL is usually a two step reaction, where in the first step the cation is synthesized by quaternisation of an amine or phosphan and in a second step the desired anion is introduced [12, 17, 18]. The most common cations and possible anions are presented in

figure 1. Since different cation/anion combinations also have different chemical and physical properties, ILs are referred to as “designer solvents” [19-23].

Common cations:



Imidazolium-Ion Pyridinium-Ion Ammonium-Ion Phosphonium-Ion

Common anions:

Cl^- Br^- NO_2^- NO_3^- BF_4^- PF_6^- $AlCl_4^-$ CF_3COO^- $CF_3SO_3^-$ $(CF_3SO_2)_2N^-$

Figure 26: Most commonly used cations and anions of ionic liquids.

The remarkable solvent properties of ILs could allow the development of a new microencapsulation technique for hydrophilic macromolecules like proteins or DNA. By discovering an IL which is capable of dissolving a biodegradable polymers and hydrophilic macromolecules a new phase separation process should be possible to encapsulate the drug substance with a minimum of degenerative forces.

The goal of this study was to screen a selection of ILs for their abilities to dissolve biodegradable polymers, proteins or DNA and trying to relate their solubility behavior to other IL characteristics. After that, a new microencapsulation technique based on biodegradable polymers and ionic liquids for hydrophilic macromolecules was developed.

Materials and methods:

Poly(DL-lactide) R202 (Mw=16 000), Poly(DL-lactide-co-glycolide) RG502 (Mw = 12 000), Poly(DL-lactide-co-glycolide) RG503 (Mw = 31 000) and Poly(DL-lactide-co-glycolide) RG505 (Mw = 80.000) were purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(DL-lactide-co-glycolide)-glucose (Mw = 55.200) was purchased from Novartis Pharma AG (Basel, Switzerland). 1-Butyl-3-methyl-imidazolium-methylsulfate ([BMIM]CH3SO4) was purchased from „Solvent Innovation“ (Cologne, Germany) and 1-Butyl-3-methyl-imidazolium-tetrafluoroborat ([BMIM]BF4) from Fluka Chemie GmbH (Buchs, Germany). All other ionic liquids from Table 1 were synthesized and provided by Novartis Pharma AG (Basel, Switzerland). All other reagents used were of analytical grade.

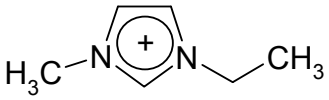
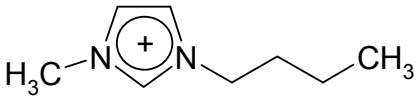
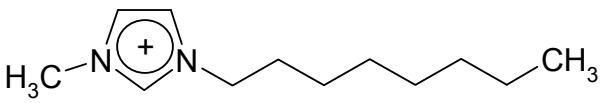
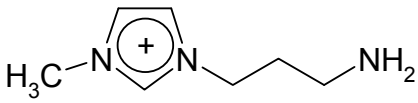
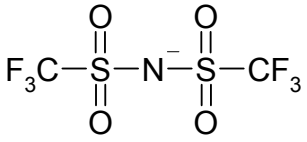
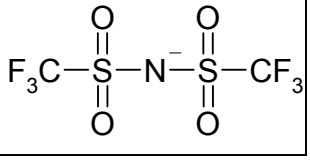
	cation	**anion**
$[EMIM]BF_4$ [EMIM]TfO [EMIM]TA		BF_4^- $CF_3SO_3^-$ CF_3COO^-
$[BMIM]BF_4$ [BMIM]TfO [BMIM]TA $[BMIM]CH_3SO_4$ $[BMIM]PF_6$		BF_4^- $CF_3SO_3^-$ CF_3COO^- CH_3SO_4 PF_6^-
[OMIM]Br		Br^-
KIL6686		
KIL6684		

Table 1: Structures of tested ionic liquids.

Solubility of biodegradable polymers in ILs

1 ml of the ionic liquids in Table 1 was added to 50 mg of the different biodegradable polymers and the mixture was homogenized by vortexing. After storing for 24 h under ambient conditions the tubes were centrifuged and the supernatant was isolated. Dissolved polymers were precipitated by the addition of water or water/ethanol mixtures for water immiscible ILs. The amount of precipitated polymer was determined gravimetrically.

Solubility of hydrophilic macromolecules in ILs

1 ml of the ionic liquids in Table 1 was added to 10 mg of the different proteins (Bovine serum albumin (BSA), Lysozyme, Cytochrome C, Insulin). After storing for 24 h under ambient conditions the tubes were centrifuged and the supernatant was isolated. Dissolved protein was precipitated by the addition of acetone. The amount of precipitated protein was determined gravimetrically. In addition, UV/Vis spectra of the supernatants were recorded using an UV/Vis spectrophotometer UV-160 (Shimadzu Corporation, Kyoto, Japan).

1 ml of several ionic liquids from Table 1 was added to 3 mg of Herring testes DNA. After storing for 24 hours under ambient conditions the tubes were centrifuged and the supernatant was isolated. UV/Vis spectra of the supernatants were recorded using an UV/Vis spectrophotometer UV-160 (Shimadzu Corporation, Kyoto, Japan).

4 µl of a pCMV-Luc solution (5mg / ml) was added to 1 ml of several ionic liquids from Table 1. After storing for 24 hours under ambient conditions the tubes were centrifuged and the supernatant was isolated. Agarose gel electrophoresis was used to check for dissolved plasmid DNA.

Size of microparticles

The average size and the size distribution of the microparticles were determined using a Malven Mastersizer X (Malvern Instruments, UK). Particles were suspended in water and constantly agitated during measurements.

Microencapsulation technique

Microencapsulation was carried out in liquid paraffin with 2% of the emulsifier Brij 93 (Uniqema Emmerich, Germany) as stabilizer for the emulsion of ionic liquids in liquid paraffin. Polyvinylalcohol Mowiol 3-83 (Hoechst AG Frankfurt am Main, Germany) was added to the aqueous phase for microparticle isolation.

Morphology of microparticles

Fragments of the nanofiber nonwovens were sputtered with gold in an argon atmosphere (0.3 mPa) using a sputter coater S-150 (Edwards Kniese & Co. GmbH, Marburg, Germany). Sputtering was carried out for each sample at 18 mA 2 times for 1 minute and after that 2 times for 2 minutes. Images were taken with an SEM S-510 at 25 kV accelerating voltage (Hitachi Ltd. Corporation, Tokyo, Japan) using the Digital Imaging Scanning System (Version 5.4.12.1) purchased from point electronic GmbH, Halle (Saale), Germany. Images were analyzed with the Digital Image Processing System (Version 2.6.13.0) purchased from point electronic GmbH, Halle (Saale), Germany.

Gel electrophoresis

Native Page gel electrophoresis experiments were carried out using a Pharmacia LKB PHAST system (Pharmacia Biosciences, Uppsala, Sweden) and PhastGel separation media for Native PAGE (Pharmacia Biosciences, Uppsala, Sweden). The different protein solutions were

applied on the ready-to-use PhastGel 18-25 (Pharmacia Biosciences, Uppsala, Sweden) via a PhastSystem applicator comb. These special sample applicators ensure that identical volumes were put on the separation gel. PhastGel Native ready-to-use buffer strips were used for the separation process. After applying current, the proteins migrated through the gradient gel and were separated according to their size. A Coomassie staining was carried out for protein detection using PhastGel Blue R tablets.

Release studies

20 mg of the lyophilized microparticles were put in vials and 5 ml of PBS buffer solution pH 7.4 was added. The vials were put into a Rotatherm that was thermostated at 37 °C and turning at 15 rpm. At several timepoints the vials were centrifuged and 1 ml of the buffer solution was removed and replaced by 1 ml of fresh buffer solution. The microparticles were resuspended and the vials put back into the Rotatherm. The amount of released substance was determined by UV/Vis-spectroscopy.

Cytotoxicity of Ionic Liquids

Cytotoxicity of [EMIM]TfO was tested using an MTT assay according to [24]. L929 cells were seeded in 96-well microtiter plates at a density of 4200 cells per well and allowed to grow for 72 hours prior to the application of the IL solutions yielding concentrations ranging from 0.3 µg/ml to 10 mg/ml of the IL in cell media. After 4 hours of incubation, the medium was replaced with 200 µL fresh medium and 20 µL (3-(4,5-dimethylthiatol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (MTT, Sigma, Seelze). After 4 hours, the unreacted dye was removed and 200 µL DMSO was added. The absorption was measured using the ELISA reader Titertek Plus MS 212 (ICN, Eschwege, Germany) at 570 nm, with a background correction at 690 nm. The relative cell viability (%) was related to control wells containing

cell culture medium without IL and was calculated by: absorption test x 100 % / absorption control. Data are presented as a mean of six measurements (± SD).

Results and discussions:

The objective of this study was to develop a new microencapsulation technique which uses an ionic liquid (IL) as one common solvent for polymer and drug. After emulsifying this solution in a lipophilic phase, the addition of water as phase separator to the emulsion should lead to precipitation of the polymer in the droplets of the inner phase of the emulsion and in addition to the encapsulation of the dissolved protein within the formed microparticles. For the development of this technique two prerequisites had to be accomplished: At first, sufficient solubility of a biodegradable polymer in the ILs and secondly, sufficient solubility of the desired drugs i.e. hydrophilic macromolecules in the ILs. Figure 2 shows the solubility of several biodegradable polymers in ILs.

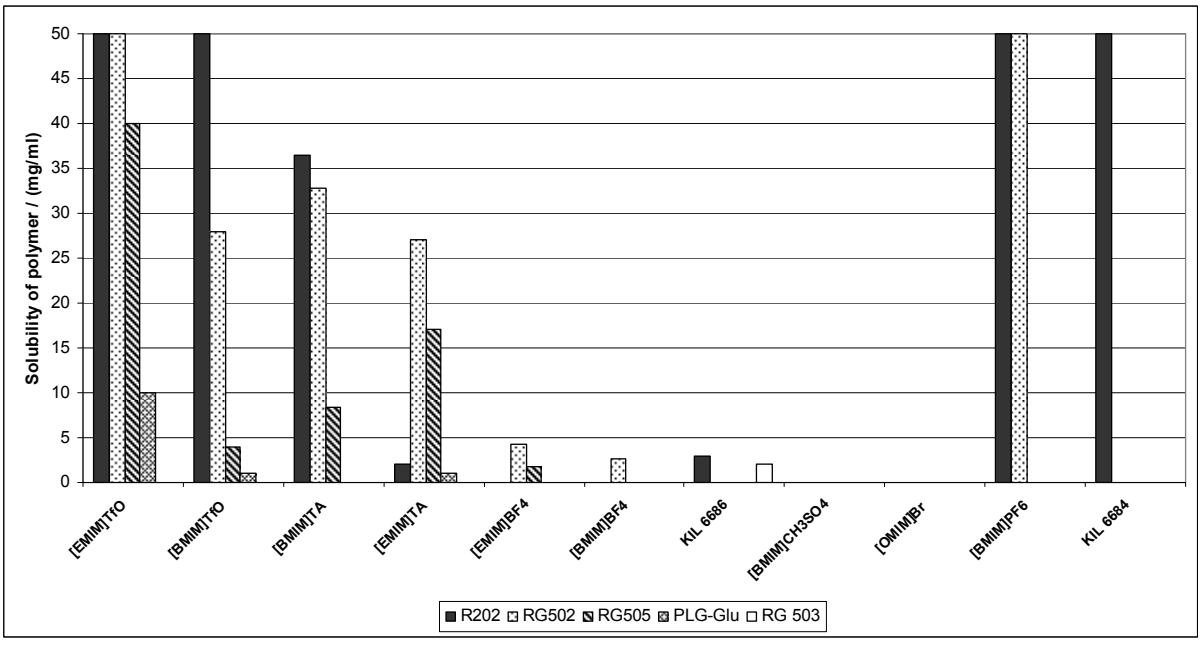


Figure 27: Most commonly used cations and anions of ionic liquids.

Some biodegradable polymers were sufficiently soluble in the ILs. The most promising among the tested ILs were composed of imidazolium cations with short alkyl chains in

combination with an anion with an acidic group. [BMIM]PF6 and KIL6684 showed also good solubility properties, but there immiscibility with water made them less suitable for our purposes. Promising ILs for further experiments were 1-ethyl-3-methyl-imidazolium-tetrafluorosulfonate [EMIM]TfO, 1-ethyl-3-methyl-imidazolium-tetrafluoroacetate [EMIM]TA, 1-butyl-3-methyl-imidazolium-tetrafluorosulfonate [BMIM]TfO and 1-butyl-3-methyl-imidazolium-tetrafluoroacetate [BMIM]TA.

4 different proteins were tested for their solubility in the ILs: bovine serum albumin (BSA), lysozyme, cytochrome C and insulin. In all cases no precipitates were detectable. Only in one case (Cytochrome C in [EMIM]TA) an evidence of dissolved protein was found in the UV/Vis-spectrum of the supernatant, but the solubility of cytochrome C in [EMIM]TA was far below 1 mg/ml. This concentration was not sufficient for our microencapsulation experiments. The obtained results were in agreement with other publications like [25], which states that the "… dissolution of proteins … in ionic liquids is generally hindered by their insolubility." In addition, the solubility of Herring testes DNA and of a plasmid DNA (pCMV-Luc) in the ILs was determined. Neither UV/Vis spectroscopy nor agarose gel electrophoresis were able to detect dissolved DNA.

Since the desired drugs are not soluble in the ILs another approach was developed to encapsulate proteins using an ionic liquid as solvent. A solution of Poly(DL-lactide) R202 in [EMIM]TfO was emulsified in liquid paraffin. The emulsifier Brij 93 was added prior to the liquid paraffin to stabilize the emulsion. Stepwise addition of an aqueous BSA solution to the emulsion led to precipitation of the polymer and formation of microparticles. The resulting suspension was then added to a vial containing water. After centrifugation and removal of the supernatant the microparticles were isolated (Figure 3).

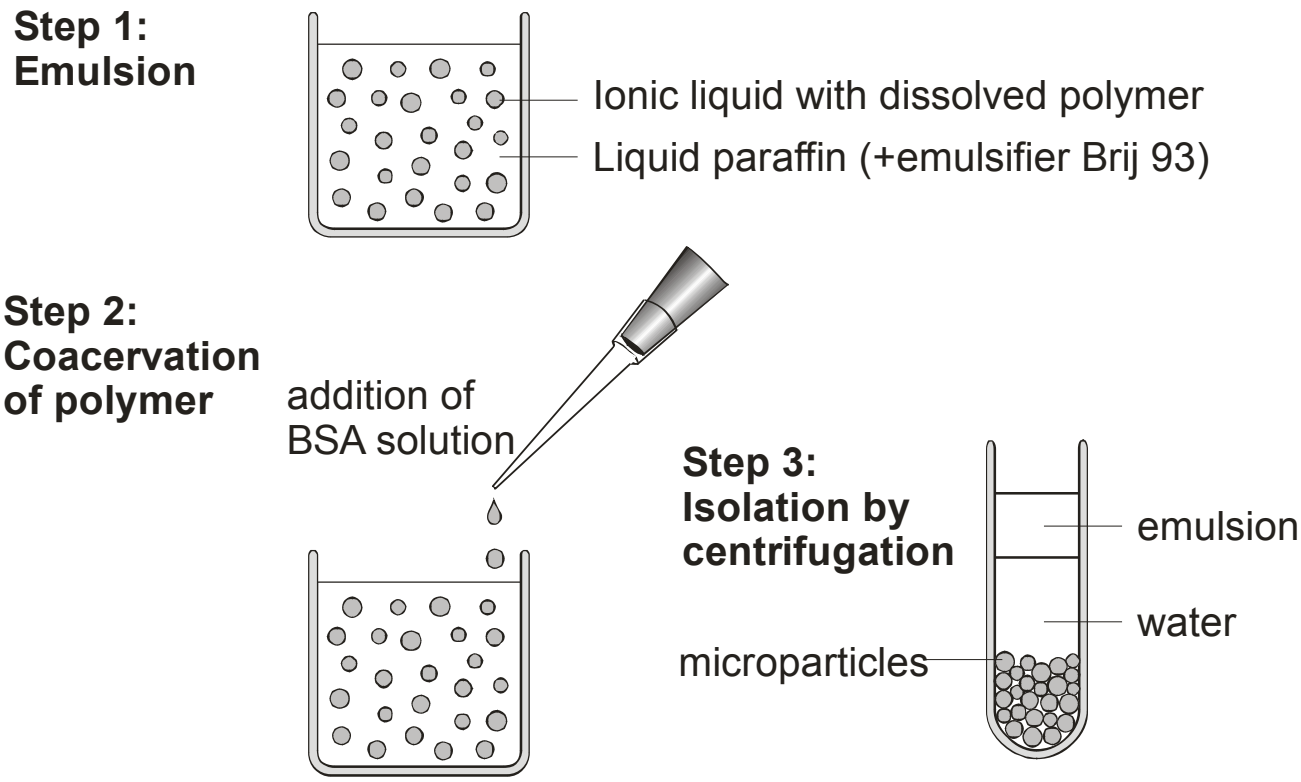


Figure 28: Processing steps of a microencapsulation technique using an ionic liquid.

Three differently concentrated polymer solutions were used: 20 mg/ml, 30 mg/ml and 40 mg/ml. After isolation of the microparticles by centrifugation they were resuspended and their size determined using a Malven Mastersizer X. The size of the microparticles was monomodally distributed. The following weighted averages of the volume distribution were found: for 20 mg/ml: D [4,3] = 32,04 µm, for 30 mg/ml: D [4,3] = 27,02 µm and for 40 mg/ml: D [4,3] = 32,02 µm. The concentration of the polymer solution did not seem to have a great influence on the resulting particle size.

SEM pictures of the microparticles were taken to evaluate the morphology of the microparticles (Figure 4). The SEM images showed microparticles with an average diameter of 20 - 30 µm. These results are in agreement with the data obtained by the above mentioned mastersizer measurements. The rough surface of the microparticles is the consequence of various washing steps which were needed to remove the ionic liquids. Using less washing

steps it was impossible to prepare SEM images of the particles due to remaining ILs. Since ILs cannot be vaporized, it is impossible to remove these substances in vacuum.

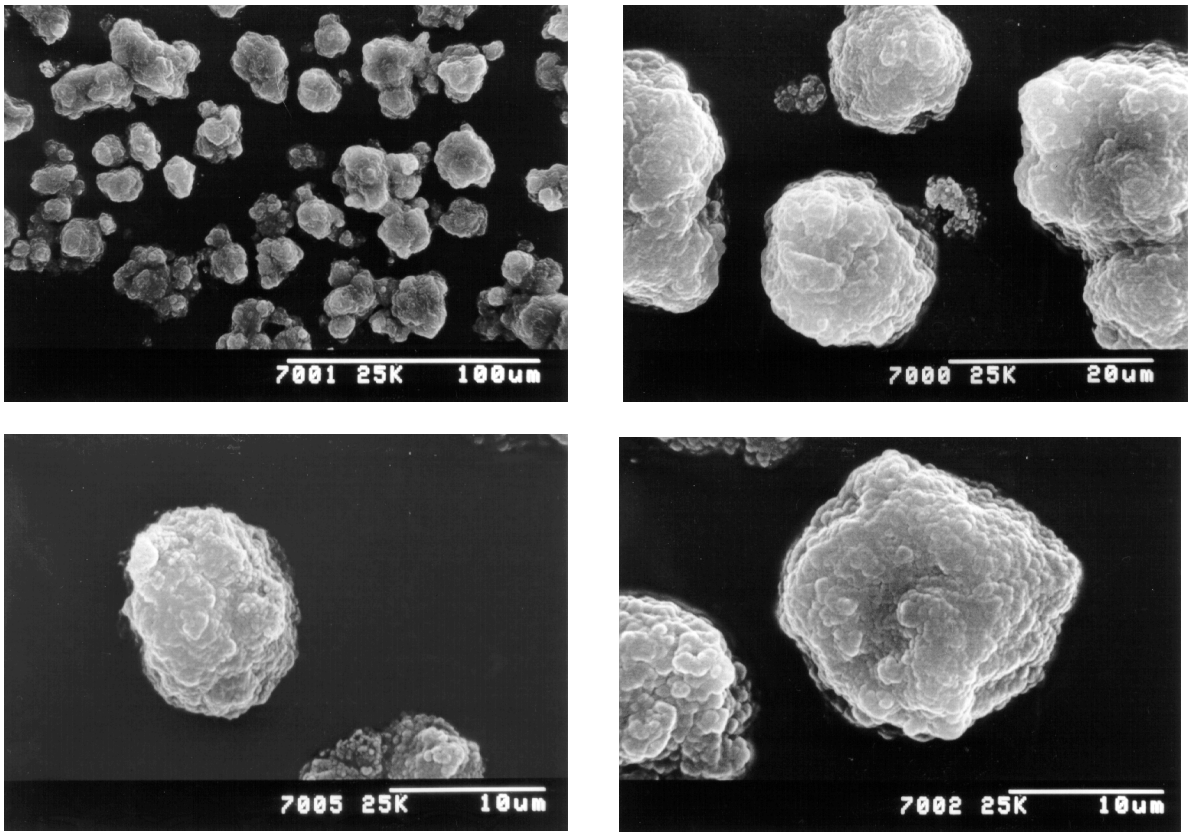


Figure 29: SEM images of microparticles prepared with an ionic liquid.

Successful encapsulation of BSA was demonstrated by dissolving the microparticles in methylene chloride, removing the supernatant and dissolving the remaining precipitant in water. The UV/Vis spectrum of the aqueous solution was similar to that of a BSA solution and native PAGE gel electrophoresis of this solution also proved that BSA was encapsulated for microparticles produced with [EMIM]TfO as solvent for the polymer. No BSA could be detected in the remaining precipitant of microparticles produced with [BMIM]TfO (Figure 5).

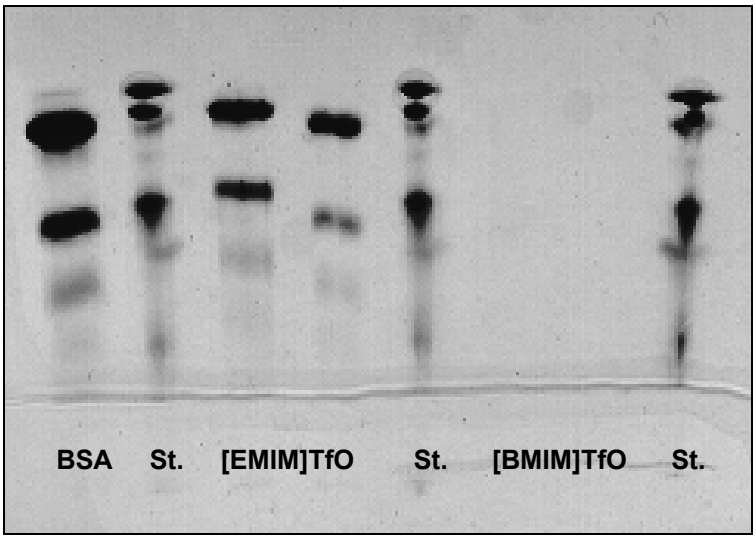


Figure 30: Native page gel electrophoresis of encapsulated BSA.

Encapsulation efficiency was determined for four different polymer solutions. The amount of BSA in the first precipitate was relatively high (Table 2), but after additional washing of the microparticles with water only small amounts of BSA were detected (Table 3). Presumably the major part of BSA was not encapsulated in the microparticles but rather precipitated outside the polymer.

c(polymer)	20 mg/ml	30 mg/ml	40 mg/ml	50 mg/ml
EE	46.85 %	52.47 %	50.23 %	65.68 %
loading	18,98 %	14,88 %	11,16 %	11,61 %

Table 2: Encapsulation efficiency and loading determined in first precipitate.

c(polymer)	20 mg/ml	30 mg/ml	40 mg/ml	50 mg/ml
EE	6,00 %	4,23 %	8,25 %	8,61 %
loading	2,82 %	1,34 %	1,95 %	1,65 %

Table 3: Encapsulation efficiency and loading determined after additional washing steps.

Since proteins and DNA are not soluble in the tested ILs these substances could only be encapsulated with low encapsulation efficiency and loading. To prove that it was possible to encapsulate substances by dissolving them and the polymer in one IL, a dye as drug model should be encapsulated. The first step was to find a dye, which is soluble in the IL. The solubility of several dyes in [EMIM]TfO, [BMIM]TfO, [EMIM]TA and [BMIM]TA was determined and Rose Bengal and Reichardt's dye demonstrated sufficient solubility. Reichardt's dye and Poly(DL-lactide) R202 were dissolved in [EMIM]TfO and the above mentioned technique was used for the encapsulation of the dye. After dissolving of the microparticles in methylene chloride the encapsulation efficiency and loading should be determined by UV/Vis-spectroscopy, but the quantitative detection via UV/Vis was impossible due to strong interactions between the chromophores of Reichardt's dye and the IL. It was possible to encapsulate Reichardts dye, but it was not possible to evaluate the amount of encapsulated dye.

In a second approach, Rose Bengal and Poly(DL-lactide) R202 were dissolved in [EMIM]TfO and the above mentioned technique was used for the encapsulation of the dye. The amount of encapsulated Rose Bengal was too low to be detectable via UV/Vis-spectroscopy. Encapsulation efficiency and loading could not be determined. The major problem was that the dye dissolved in the added phase separator (water) before the polymer precipitated. After that, several experiments were carried out to increase loading and encapsulation efficiency by variation of the encapsulation method: The concentration of the polymer was varied in the range of 20 - 50 mg/ml, different phase separators (e.g. methanol, ethanol, isopropanol or other ionic liquids) were used and other polymers were dissolved in the IL (e.g. RG503, RG505). None of these variations led to an increase in encapsulation efficiency. But the addition of an aqueous solution of Rose Bengal (20 mg/ml) as phase separator to an IL solution of poly(DL-lactide) emulsified in liquid paraffin led to intensely

red colored particles. 3 different polymer solutions and 2 different Rose Bengal solutions were used to produce microparticles by the above mentioned method (Table 4).

c(RB) / mg/ml	c(R202) / mg/ml	loading / w%	EE / %
10	20.52	4.92	23.97
20	20.05	9.34	46.58
10	30.59	5.19	16.96
20	30.83	12.25	39.73
10	40.16	5.62	13.99
20	40.53	13.04	32.17

Table 4: Loading and encapsulation efficiency of microparticles produced with 3 different polymer solutions and 2 different RB solutions.

The encapsulation efficiency decreased with an increasing concentration of the polymer and increased with an increasing dye concentration. The size of the microparticles was monomodally distributed with weighted averages of the volume distribution ranging between 30 and 55 µm. These microparticles were bigger than the ones prepared with BSA solution, which had diameters of ca. 30 µm.

Figure 6 shows the release profile of Rose Bengal from the microparticles. After 3 hours 100% of the dye was released from the microparticles. The release profile lets suggest, that only absorption of the dye to the surface of the microparticles occurred. Since Rose Bengal is likely to absorb on polymer surfaces probably only a very small amount of Rose Bengal was actually encapsulated inside the microparticles.

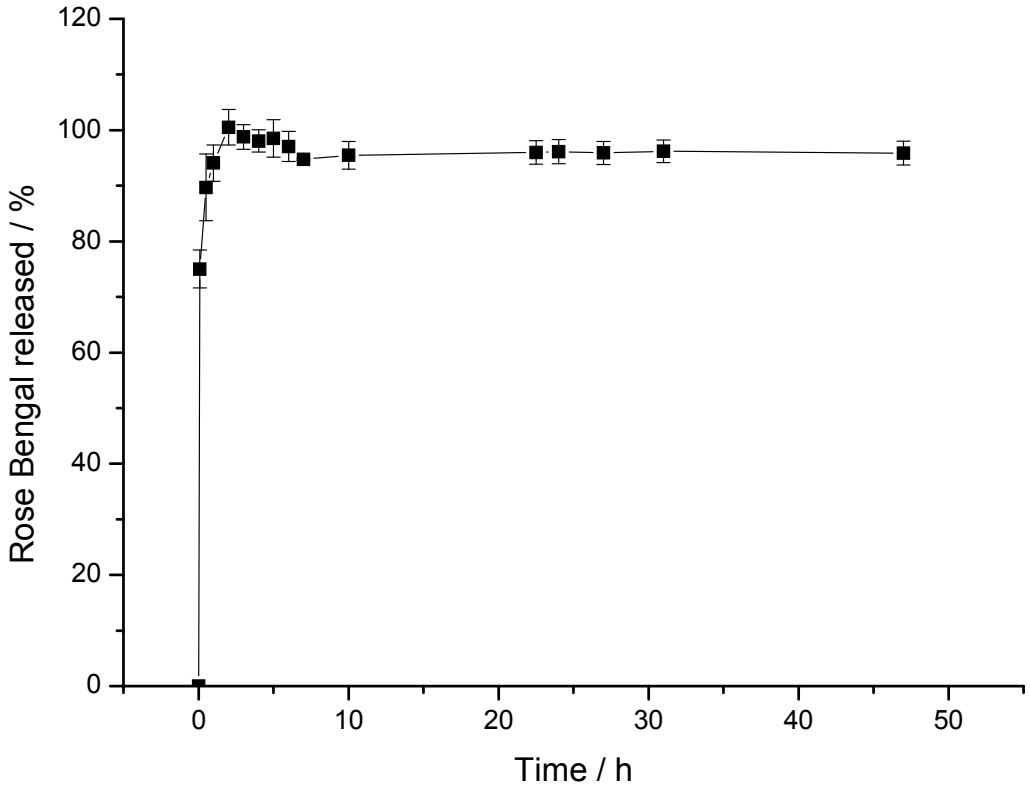


Figure 31: Release profile of Rose Bengal from microparticles prepared using an ionic liquid.

Another dye should be chosen which shows sufficient solubility in the ILs, but is only slightly soluble in water and insoluble in liquid paraffin. The most important point was the solubility of the dye in water. The substance had to be at least slightly soluble in water to determine its release profile in an aqueous buffer solution. But the dye should not dissolve in the water, which was added as phase separator during the production of the microparticles. Neither should it be too lipophilic since then it would dissolve in the liquid paraffin. Furthermore, the ILs had made the search for an adequate dye more difficult, because they had a solubilizing effect in water. This meant a dye that was only slightly soluble in water dissolved easily by addition of small amounts of an IL. 17 different dyes were tested and among them thymol blue seemed to match the above mentioned properties best. Encapsulation of thymol blue was carried out by dissolving 50 mg R202 and 8 mg TB in 1 ml [EMIM]TfO, emulsification of this solution in liquid paraffin with 1 % Brij 93 and the addition of water as phase separator.

The loading of the microparticles was very low with values less than 1%. Again the dye was dissolved in the phase separator supported by the solubilizing properties of the ILs during the preparation of the microparticles.

Although the ILs have to be removed completely from the prepared microparticles, these substances should feature a low cytotoxicity to be suitable for the preparation of parenteral dosage forms. An MTT assay was carried out to determine the cytotoxicity of [EMIM]TfO. The tested concentrations ranged between 0.3 µg/ml and 10 mg/ml. For concentrations below 5 mg/ml only slight influence on cell viability could be detected (Figure 7). It is most unlikely that the amount of remaining IL in the microparticles will lead to such high concentrations of ILs in vivo. This leads to the conclusion that ILs are suitable solvents for the production of microparticles.

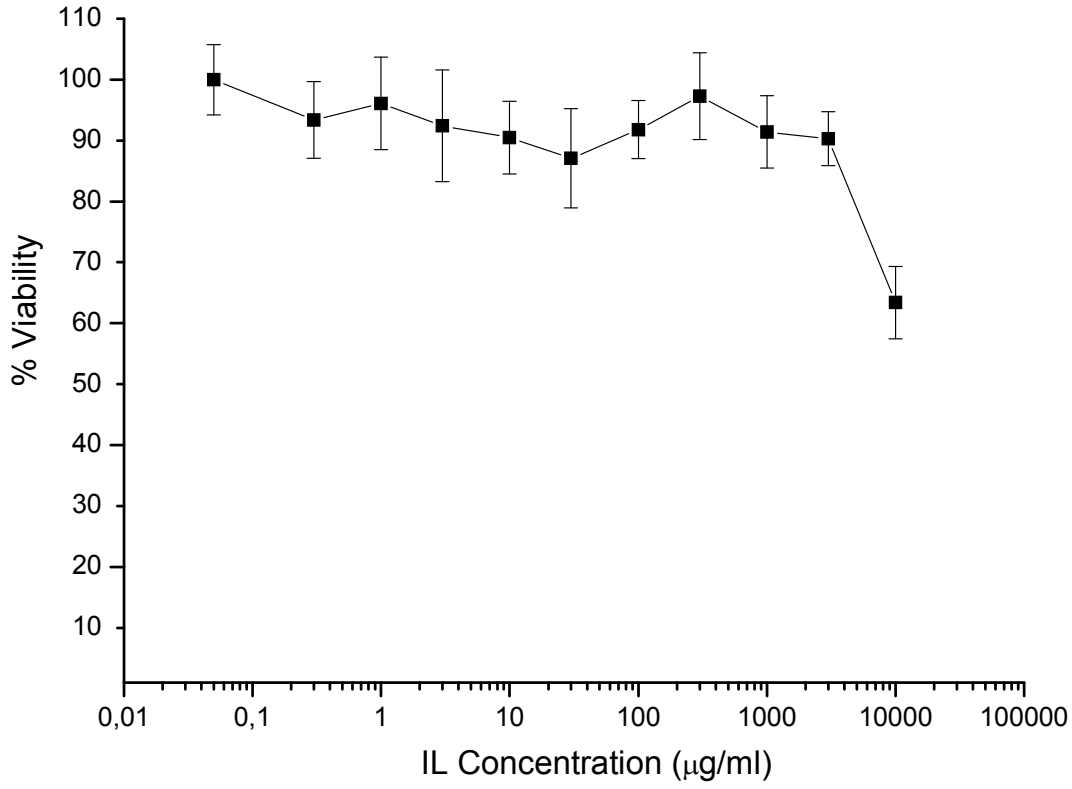


Figure 32: Cytotoxicity of the ionic liquid [EMIM]TfO.

Conclusion:

We demonstrated that biodegradable Polymers were sufficiently soluble in ILs, but hydrophilic macromolecules did not show sufficient solubility. Nevertheless the microencapsulation of BSA was possible via a modified technique but showed very low encapsulation efficiency. The size of the microparticles ranged between 25 - 30 µm but the morphology remained unclear, since several washing steps, which influenced the morphology of the microparticles, were needed to prepare them for SEM imaging. Reichardts dye was soluble in ILs and was encapsulated, but could not be quantified via UV/Vis-spectroscopy. Rose Bengal was slightly soluble in ILs but dissolved in the added phase separator during preparation of microparticles, which led to low encapsulation efficiencies. The release profile lets suggest, that only absorption of the dye on the surface of the mircoparticles occurred. Thymol blue was soluble in ILs and only slightly soluble in water, but as a result of the solubilizing effect of the ILs the major part also dissolved in the added phase separator during preparation of the microparticles. The low cytotoxicity of [EMIM]TfO proved that ILs are suitable solvents for the preparation of microparticles. Although we were not able to find an IL among the tested ones, which is capable of dissolving a biodegradable polymer and a hydrophilic macromolecule, this goal might be easily achievable with another IL. J.D. Holbrey und K.R. Seddon assumed that there are about 10^{18} possible cation / anion combinations which yield an IL [1] and it is most likely that one amongst them has the suitable solution properties for our purposes.

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SUMMARY AND PERSPECTIVES

Summary and Perspectives:

This thesis describes two novel technologies for the encapsulation of proteins in biodegradable polymers. Protein loaded nanofiber nonwovens and microparticles were manufactured and characterized regarding their suitability as drug delivery devices.

Chapter 1 gave a detailed overview on the current status of electrospinning as a technique for the generation of nanofibrous scaffolds. Basic principles of electrospinning as well as several modification of the standard method were discussed and the applications of electrospun scaffolds in the biomedical field were elucidated.

Several biodegradable polymers (Poly(L-lactide) (PLLA) Resomer L210, poly(lactide-co-glycolide) (PLGA) Resomer RG858 and Poly(ethylene carbonate) (PEC)) were investigated in **Chapter 2** for their application in electrospinning of emulsions. Suitable process parameters were determined for all polymers to generate protein loaded nanofiber nonwovens (NNs). The resulting NNs were examined regarding their morphology and regarding their protein release profile. PEC formed the most uniform fibers with diameters of 1 to 5 µm. PLLA and PLGA formed less uniform fibers but with smaller diameters of 0.3 to 1 µm. PEC and PLGA NNs showed only burst release of the incorporated protein and no further release within 2 weeks. PLLA NNs released the protein very slowly and in a controlled manner. Due to the swelling of PLGA and PEC almost all fibers had lost their individuality after incubation in buffer solution. The morphology of PLLA NNs was more or less unchanged after the release study. PLLA NNs exhibited the most promising characteristics and hence were chosen for further studies.

In **Chapter 3** protein loaded PLLA NNs were electrospun from differently concentrated solutions. The NNs featured a superhydrophobic surface and it was demonstrated, that the wettability of these scaffolds was the major factor influencing the protein release. Although TEM images showed that the major part of the protein was most likely located between the

fibers and not encapsulated inside the fibers, the protein was released very slowly from the PLLA NNs. Only 20 – 30% of the protein was released within one month. The addition of hydrophilic polymers like poly(ethylene imine) (PEI) or poly(L-lysine) (PLL) to the aqueous phase of the electrospinning emulsion yielded NNs composed of polymer blends of PLLA and the respective hydrophilic polymer. It was demonstrated that the amount of added hydrophilic polymer had a significant effect on the release profile. A higher amount of hydrophilic polymer led to a faster release of the protein and gave us the opportunity to tailor the release profile of these NNs. Compositions of 5 or 10% PLL or PEI and 95 or 90% PLLA respectively, showed the most promising release profiles. Further screening of compositions ranging from 1 to 10% of hydrophilic polymers should be investigated to find the most appropriate release pattern.

Whether NNs electrospun from emulsions are suitable scaffolds for their application in tissue engineering was determined in **Chapter 4**. The prepared NNs met all requirements for a tissue engineering scaffold: Mean fiber diameters of the scaffolds were very similar to fibrous protein structures of the extracellular matrix (ECM), meaning these scaffolds could physically resemble the ECM. Cell adhesion, cell proliferation and cell spreading on all NNs were at least as good as on a glass surface. For a composition of 5% PLL and 95% PLLA these values were even significantly higher. NNs took up water and swelled in the cell medium. The extent of swelling depended on the amount of hydrophilic polymer with a higher fraction leading to increased swelling. Cells still adhered to the fibers and were able to proliferate in all three dimensions. There were no significant differences in cell viability for cells grown on NNs compared to the cell viability on a glass surface. These results strongly suggest a huge potential of these nanofiber nonwovens for their application in tissue engineering.

Chapter 5 gave an overview on microencapsulation focusing on phase separation and coacervation techniques. The development of these techniques and its application in the pharmaceutical industry were elucidated.

The development of a new microencapsulation technique which utilizes an ionic liquid as one common solvent for a biodegradable polymer and a hydrophilic macromolecule was presented in **Chapter 6**. Some of the tested ionic liquids were good solvents for biodegradable polymers, but none of them was able to dissolve hydrophilic macromolecules. Even though there was no suitable ionic liquid among the tested compounds, the probability of finding a suitable ionic liquid is very high, since there are almost countless possibilities for the synthesis of different ionic liquids. Nevertheless the feasibility of encapsulating BSA via an emulsion based phase separation technique was demonstrated.

Generally, it can be stated that the presented novel techniques for the encapsulation of proteins in biodegradable polymers demonstrated their huge potential for the preparation of drug delivery devices. Protein loaded nanofiber nonwovens exhibited very promising properties for their use as tissue engineering scaffolds and will definitely find application in this field. Screening the huge library of ionic liquids to find a compound with suitable solvent properties will be a difficult task, but the discovery of an appropriate ionic liquid will be a powerful tool for the encapsulation of proteins in microparticles

ZUSAMMENFASSUNG UND AUSBLICK

Zusammenfassung und Ausblick:

Diese Doktorarbeit beschreibt zwei neuartige Technologien für die Verkapselung von Proteinen in bioabbaubaren Polymeren. Nanofasermatten und Mikropartikel, die mit Proteinen beladen waren, wurden hergestellt und auf ihre Anwendbarkeit als Systeme für kontrollierte Wirkstofffreigabe getestet.

Kapitel 1 gibt einen detaillierten Überblick über den aktuellen Stand über die Anwendung des Elektrospinnens zur Herstellung von Nanofasermatten. Sowohl grundlegende Prinzipien des Elektrospinnens als auch einige Modifikationen der Standardmethode werden diskutiert und die Anwendung von elektrogesponnen Nanofasermatten im Bereich der Biomedizin werden erläutert.

Mehrere bioabbaubare Polymere (Polymilchsäure (PLLA) Resomer L210, Polylaktid-co-glykolid (PLGA) Resomer RG858 und Polyethylencarbonat (PEC)) werden in Kapitel 2 auf ihre Anwendbarkeit für das Elektrospinnen von Emulsionen untersucht. Geeignete Prozessparameter wurden für alle Polymere ermittelt, um mit Protein beladene Nanofasermatten (NNs) herzustellen. Die erhaltenen NNs wurden bezüglich ihrer Morphologie und ihres Protein Freisetzungsprofils charakterisiert. PEC bildete die gleichmäßigsten Fasern mit Durchmessern von 1 – 5 μm. PLLA und PLGA Fasern waren weniger gleichmäßig, zeigten aber kleinere Durchmesser von 0,3 – 1 μm. PEC und PLGA NNs zeigten nur eine schnelle initiale Proteinfreisetzung und keine weitere Freisetzung während der folgenden 2 Wochen. PLLA NNs gaben das Protein sehr langsam und gleichmäßig ab. Aufgrund der Quellung von PLGA und PEC hatten fast alle Fasern ihre Individualität verloren, nachdem sie in Pufferlösung inkubiert worden waren. Die Morphologie der PLLA NNs war mehr oder weniger unverändert nach der Freisetzungsstudie. PLLA NNs zeigten die verheißungsvollsten Eigenschaften und wurden daher für weitergehende Versuche ausgewählt.

In Kapitel 3 wurden mit Protein beladene NNs aus unterschiedlich konzentrierten Polymerlösungen electrogesponnen. Die NNs hatten eine superhydrophobe Oberfläche und es konnte gezeigt werden, dass die geringe Benetzbarkeit dieser Fasermatten den größten Einfluss auf die Proteinfreisetzung hat. Obwohl TEM Bilder gezeigt hatten, dass ein großer Teil des Proteins wahrscheinlich zwischen den Fasern vorliegt und nicht in ihnen verkapselt ist, wurde das Protein nur sehr langsam aus den NNs freigesetzt. Nur 20 – 30% des Proteins wurde innerhalb eines Monats freigesetzt. Die Zugabe von hydrophilen Polymeren wie Polyethylenimin (PEI) oder Poly-L-lysin (PLL) zur wässrigen Phase der Electrospinning-emulsion ergab NNs die aus einer Polymermischung von PLLA und dem jeweiligen hydrophilen Polymer bestanden. Es konnte gezeigt werden, dass die Menge an zugegebenem hydrophilem Polymer einen signifikanten Einfluss auf das Freisetzungsprofil hat. Ein größerer Anteil an hydrophilem Polymer führte zu einer schnelleren Freisetzung des Proteins und ermöglichte dadurch, das Freisetzungsprofil der NNs entsprechend einzustellen. Zusammensetzungen aus 5 oder 10% PLL oder PEI und entsprechend 95 oder 90% PLLA zeigte die aussichtsreichsten Freisetzungsprofile. Eine weitere Abklärung der Freisetzungsprofile von Zusammensetzungen mit 1 bis 10% an hydrophilem Polymer sollten noch genauer untersucht werden, um das am besten geeignete Freisetzungsprofil zu finden.

Ob aus Emulsionen gesponnene NNs geeignete Substrate für eine Anwendung als Tissue engineering scaffold sind wird in Kapitel 4 untersucht. Die hergestellten NNs erfüllten alle Voraussetzungen für ein Tissue engineering scaffold: der mittlere Faserdurchmesser der NNs war den fibrösen Proteinstrukturen der extrazellulären Matrix (ECM) sehr ähnlich; dies bedeutet, das diese NNs die ECM physikalisch imitieren können. Zelladhäsion, Zellproliferation und die Zellspreitung auf den NNs war mindestens genauso gut wie auf einer Glasoberfläche. Für eine Zusammensatzung von 5% PLL und 95% PLLA waren diese Werte sogar signifikant erhöht. Die NNs nahmen Wassser auf und quollen im Zellmedium. Das

Ausmaß der Quellung war abhängig von der Menge an hydrophilem Polymer und ein höherer Anteil führte zu einer erhöhten Quellung. Die Zellen adherierten weiterhin an den Fasern und bekamen dadurch die Möglichkeit in alle drei Dimensionen zu proliferieren. Es gab keine signifikanten Unterschiede in der Zellviabilität zwischen Zellen, die auf NNs gewachsen waren verglichen mit Zellen, die auf einer Glasoberfläche gewachsen waren. Diese Ergebnisse zeigen das große Potential dieser NNs für ihre Anwendung im Bereich des Tissue engineerings.

Kapitel 5 gibt einen Überblick über die Mikroverkapselung mit dem Fokus auf Phasenseparations- und Koazervationsverfahren. Die Entwicklung dieser Techniken und ihre Anwendungen in der pharmazeutischen Industrie werden erläutert.

Die Entwicklung eines neuen Mikroverkapselungsverfahrens, bei dem eine ionische Flüssigkeit als gemeinsames Lösungsmittel sowohl für ein bioabbaubares Polymer als auch für ein hydrophiles Makromolekül verwendet wird, wird in Kapitel 6 beschrieben. Einige der getesteten ionischen Flüssigkeiten waren gute Lösungsmittel für bioabbaubare Polymere, aber keine von ihnen war in der Lage hydrophile Makromoleküle zu lösen. Obwohl unter den getesteten ionischen Flüssigkeiten keine geeignete Verbindung war, ist die Wahrscheinlichkeit eine geeignete Substanz zu finden sehr hoch, da es beinahe unzählige Möglichkeiten für die Synthese unterschiedlicher ionischer Flüssigkeiten gibt. Trotzdem konnte gezeigt werden, dass es möglich ist BSA über eine emulsionsbasierte Phasenseparationstechnik zu verkapseln.

Im Allgemeinen kann gesagt werden, dass die hier präsentierten neuartigen Methoden zur Verkapselung von Proteinen in bioabbaubaren Polymeren ihr großes Potential zur Herstellung von Systemen für kontrollierte Wirkstofffreigabe gezeigt haben. Mit Proteinen beladene Nanofasermatten zeigten sehr vielversprechende Eigenschaften für ihre Anwendung als Tissue engineering scaffolds und werden mit Sicherheit in diesem Feld eine Anwendung

finden. Das Durchsuchen der riesigen Auswahl an ionischen Flüssigkeiten, um eine Verbindung mit geeigneten Lösemitteleigenschaften zu finden, mag eine schwierige Aufgabe sein, allerdings hätte man mit der Entdeckung einer geeigneten Substanz ein mächtiges Werkzeug für die Verkapselung von Proteinen zur Hand.

APPENDICES

Angaben zur Person

Name	Sascha Maretschek
Staatsangehörigkeit	Deutsch
Geburtsdatum und Ort	23.08.1976 in Fulda

Schulausbildung

1983 – 1987	Grundschule Eichenzell
1987 – 1996	Rabanus-Maurus-Schule (Domgymnasium) Fulda Humanistisches Gymnasium mit neusprachlichem Zweig
25.06.1996	- Erteilung der Allgemeinen Hochschulreife -

Zivildienst

1996 – 1997	Behindertenbetreuung für die Caritas-Einrichtung der Behindertenhilfe Fulda

Hochschulstudium

1997 – 2001	Studium der Pharmazie an der Philipps-Universität Marburg
16.12.2002	- Erteilung der Approbation als Apotheker -

Berufstätigkeit

2003 – 2008	Wissenschaftlicher Mitarbeiter am Institut für pharmazeutische Technologie und Biopharmazie der Philipps-Universität Marburg im Arbeitskreis von Herrn Prof. Dr. Kissel Anfertigung einer Diplomarbeit mit dem Titel: „Untersuchungen zu neuen Mikroverkapselungsmethoden basierend auf bioabbaubaren Polymeren und ionischen Flüssigkeiten“ Promotion mit dem Forschungsschwerpunkt „Electrospinning von Emulsionen zur Herstellung bioabbaubarer Fasermatten für die kontrollierte Freisetzung von Proteinen“
03.07.2003	- Erlangen des akademischen Grades „Diplompharmazeut“ -

Praktika

September 1998	Famulatur in der Bahnhof-Apotheke OHG, Fulda
März 1999	Famulatur in der Apotheke des städtischen Klinikums Fulda
Nov. 2001 – April 2002	Praktikum am Institut für pharmazeutische Technologie und Biopharmazie der Philipps-Universität Marburg
Mai 2002 – Okt. 2002	Praktikum in der „Apotheke am Ludwigsplatz“ Gießen

Publikationen

“Microencapsulation techniques for parenteral depot systems and their application in the pharmaceutical industry“ T. Kissel, S. Maretschek, C. Packhaeuser, J. Schnieders, N. Seidel; Drugs and the Pharmaceutical Sciences Vol. 158 „Microencapsulation 2nd edition“ (2006) pp: 99-122

“Electrospun biodegradable nanofiber nonwovens for controlled release of proteins” S. Maretschek, A. Greiner, T. Kissel; Journal of controlled Release (2008) 127(2): p. 180-187

Tagungsbeiträge

“Electrospun biodegradable fiber mats as protein delivery device”, Controlled Release Society German Chapter Annual meeting 2005, Marburg

“Electrospinning of emulsions to generate biodegradable, protein containing scaffolds for tissue engineering and drug delivery”, Controlled Release Society German Chapter Annual meeting 2006, Jena (Auszeichnung als “Bestes Poster”)

“Nanofiber composites for the controlled release of proteins”, 33rd Annual Meeting & Exposition of the Controlled Release Society 2006, Wien

“Study of surface properties of electrospun fiber mats using gas adsorption and examination of their interactions with fibroblasts”, Controlled Release Society German Chapter Annual meeting 2007, Freiburg